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Prevalence of *Clostridium difficile* in Retail Meat and Controlling its Virulence using Carvacrol and Trans-cinnamaldehyde

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Prevalence of *Clostridium difficile* in Retail Meat and Controlling its Virulence Using

Carvacrol and *Trans*-cinnamaldehyde

Shankumar Mooyottu, PhD

University of Connecticut, 2016

Clostridium difficile is a significant pathogen causing a toxin-mediated enteric disease in humans and animals. The incidence and severity of *C. difficile* associated disease (CDAD) in humans have increased in the US with the emergence of hypervirulent strains and community associated outbreaks. The detection of genotypically similar and identical *C. difficile* strains implicated from human infections in foods and food animals suggest the potential role of food as a source of community-associated *C. difficile* disease. *C. difficile* predominantly affects hospital inpatients undergoing prolonged antibiotic therapy, which results in enteric dysbiosis, leading to *C. difficile* spore germination, pathogen colonization in the intestine and subsequent toxin production. Therapeutic agents that inhibit critical *C. difficile* virulence factors such as toxin production, sporulation, and spore outgrowth without causing enteric dysbiosis could improve the clinical outcome of *C. difficile* infections and prevent relapse of the infection. In this Ph. D. research work, the efficacy of two plant-derived molecules, carvacrol (CR) and *trans*-cinnamaldehyde (TC), were investigated for reducing *C. difficile* toxin production. Gene expression and cell culture studies were performed to elucidate the mechanisms behind the anti-toxigenic mechanism of CR and TC. Moreover, the efficacy of CR on *C. difficile* sporulation, germination, and spore outgrowth was also investigated. Furthermore, the effect of CR on CDAD pathogenesis was investigated in a mouse model with special reference to its effect on the mouse

gut microbiome. The results revealed that sub-inhibitory concentrations (SIC) of CR and TC reduced CD toxin production and cytotoxicity *in vitro* by downregulating toxin production genes ($p < 0.5$). In addition, CR inhibited *C. difficile* sporulation and spore outgrowth. Oral supplementation of CR improved the clinical outcome in the *C. difficile* challenged mice, and positively altered the gut microbiome composition ($p < 0.5$). Furthermore, the potential food transmission of *C. difficile* was investigated, where beef, pork and chicken samples obtained from geographically distant grocery stores in Connecticut were tested for *C. difficile*. Presumptive *C. difficile* isolates were characterized by ribotyping, antibiotic susceptibility, toxin production and whole genome sequencing. The results revealed that *C. difficile* occurs at very low levels in raw meat sold in Connecticut, but the isolates were resistant to multiple antibiotics.

**Prevalence of *Clostridium difficile* in Retail Meat and Controlling its Virulence using
Carvacrol and *Trans*-cinnamaldehyde**

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2016

APPROVAL PAGE

Doctor of Philosophy Dissertation

**Prevalence of *Clostridium difficile* in Retail Meat and Controlling its Virulence using
Carvacrol and *Trans*-cinnamaldehyde**

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TABLE OF CONTENTS

Approval page.....	i
Acknowledgements.....	ii
Table of contents.....	iv
List of figures.....	ix
List of tables.....	xi
List of abbreviations.....	xii
Chapter I: Introduction.....	1
Chapter II: Review of Literature.....	6
1. <i>Epidemiology of C. difficile infection:</i>	7
2. <i>C. difficile infection and pathogenesis</i>	9
2.1. <i>C. difficile associated disease (CDAD) in humans</i>	9
2.2. <i>Pseudomembranous Colitis</i>	9
2.3. <i>Pathogenesis C. difficile associated disease (CDAD)</i>	10
2.4. <i>C. difficile toxins</i>	11
2.5. <i>Regulation of C. difficile toxin production</i>	13
3. <i>C. difficile sporulation</i>	14
3.1 <i>C. difficile spores</i>	14
3.2 <i>Regulation of C. difficile sporulation</i>	16
3.3. <i>difficile spore germination</i>	16
4. <i>C. difficile and gut microbiome</i>	17

5. Treatment strategies of <i>C. difficile</i> infection in humans.....	19
5.1 Antibiotic therapy.....	19
5.2 Fecal microbial transplantation.....	20
5.3 Vaccination.....	22
5.4 Probiotics.....	22
5.5 Alternative therapeutic strategies against CDAD.....	23
6. Plant-derived phenols	25
6.1 Carvacrol.....	25
6.2 Trans- cinnamaldehyde.....	26
7. Potential food borne transmission of <i>C. difficile</i>	27
7.1 Food animals as a source of <i>C. difficile</i>	27
7.2. Animal derived foods as a source of <i>C. difficile</i>	29
References.....	33

Chapter III: The prevalence of *C. difficile* in retail meat sold in Connecticut, and characterization of a *C. difficile* meat isolate by phenotypic and genotypic tests.

Abstract.....	79
1. Introduction.....	80
2. Materials and Methods.....	81
2.1. Sample collection and detection of <i>C. difficile</i> in meat.....	81
2.2. Detection of toxins.....	82
2.3. Antibiotic susceptibility tests	82
2.4. Toxin gene detection and ribotyping.....	83

2.5. Whole genome sequencing.....	84
3. Results and discussion.....	85
4. Conclusion.....	90
References.....	91

Chapter IV: Effect of carvacrol (CR) and *trans*-cinnamaldehyde (TC) on *C. difficile* toxin production, cytotoxicity and toxin gene expression

Abstract.....	101
1.Introduction.....	102
2. Materials and Methods.....	104
2.1. Bacterial Strains and Culture Conditions.....	104
2.2. Plant Compounds and SIC Determination.....	104
2.3. Effect of Plant Compounds on <i>C. difficile</i> Toxin Production and Cytotoxicity.....	105
2.4. ELISA for Total Toxin A and B.....	105
2.5 Cytotoxicity Assay.....	106
2.6. Real Time Quantitative PCR (RT-qPC.....	106
2.7. Construction of a <i>codY</i> Mutant of <i>C. difficile</i> Strain UK-1 and the Effect of Plant Compounds on <i>codY</i> Mutant and Parental Strains.....	107
2.8. Statistical Analysis.....	108
3. Results.....	109
3.1. Sub-Inhibitory Concentrations of CR and TC	109
3.2 Effect of CR and TC on <i>C. difficile</i> Toxin Production.....	109
3.3. Effect of CR and TC on <i>C. difficile</i> Toxin-Mediated Cytotoxicity.....	109
3.4. Effect of CR and TC on <i>C. difficile</i> Toxin Genes.....	110

3.5. <i>Effect of CR and TC on a codY Mutant of C. difficile</i>	111
4. Discussion.....	111
5. Conclusion.....	114
References.....	116
Chapter V: Effect of Carvacrol on <i>C. difficile</i> sporulation and spore outgrowth	
Abstract.....	133
1. Introduction.....	134
2. Materials and Methods.....	135
2.1. <i>Bacterial strains and culture conditions</i>	135
2.2. <i>C. difficile Spore preparation</i>	135
2.3. <i>Carvacrol and determination of MIC and SIC</i>	136
2.4. <i>Effect of CR on C. difficile growth and sporulation kinetics</i>	136
2.5. <i>Real-time Quantitative PCR (RT-qPCR)</i>	137
2.6. <i>Effect of SIC and MIC of CR on C. difficile spore germination and outgrowth</i>	137
2.7. <i>Statistical analysis</i>	138
3. Results.....	138
3.1. <i>Sub-inhibitory concentrations of CR</i>	138
3.2. <i>Effect of CR on C. difficile growth and sporulation kinetics</i>	139
3.3. <i>Effect of CR on C. difficile spore germination and outgrowth</i>	139
3.4. <i>Effect of CR on C. difficile spore production genes</i>	140
4. Discussion.....	141
References.....	144

Chapter VI: Protective Effect of Carvacrol against Gut Dysbiosis and *Clostridium difficile* Associated Disease in a Mouse Model

Abstract.....	158
1. Introduction.....	160
2. Materials and Methods.....	162
2.1. <i>Ethics statement, animals, and housing</i>	162
2.2. <i>Mouse model of C. difficile infection and treatment groups</i>	162
2.3. <i>DNA extraction, PCR amplification, and sequencing of taxonomic marker</i>	163
2.4. <i>Sequence analysis</i>	164
2.5. <i>Statistical analysis</i>	165
3. Results.....	165
3.1. <i>Effect of CR supplementation on the incidence of diarrhea and severity of C. difficile infection in mice</i>	165
3.2. <i>Effect of CR supplementation on clinical score and body weight of C. difficile infected mice</i>	166
3.3. <i>Effect of CR supplementation on the gut microbiome of C. difficile infected and non-infected mice</i>	167
4. Discussion.....	169
References.....	174
Chapter VII: Summary	202

List of Figures

	Title	Page
Chapter III		
FIG. 1	Phylogenetic tree constructed by genome blast (NCBI) demonstrating relationship between the pork isolate SG-12 and other complete and draft genomes.	99
Chapter IV		
FIG. 1	Effect of Sub-inhibitory concentrations of carvacrol (CR) and <i>trans</i> -cinnamaldehyde (TC) on beneficial gut bacterial growth.	124
FIG. 2	Effect of carvacrol (CR) and <i>trans</i> -cinnamaldehyde (TC) on <i>C. difficile</i> toxin production	125
FIG. 3	Effect of carvacrol (CR) and <i>trans</i> -cinnamaldehyde (TC) on <i>C. difficile</i> induced cytotoxicity on Vero cells.	126
FIG. 4	Effect of carvacrol (CR) on <i>C. difficile</i> toxin regulatory genes.	128
FIG. 5	Effect of <i>trans</i> -cinnamaldehyde (TC) on <i>C. difficile</i> toxin regulatory genes	129
FIG. 6	Effect of carvacrol (CR) and <i>trans</i> -cinnamaldehyde (TC) on codY mutant and wild type <i>C. difficile</i> toxin production.	130
FIG. 7	Effect of carvacrol (CR) and <i>trans</i> -cinnamaldehyde (TC) on codY mutant and wild type <i>C. difficile</i> toxin genes	131
Chapter V		
FIG. 1	Effect of carvacrol (CR) on <i>C. difficile</i> BAA 1870 (1A) and 1805 (1B) growth and sporulation kinetics	150

FIG. 2	Effect of carvacrol (CR) on <i>C. difficile</i> ATCC BAA 1870 and BAA 1805 sporulation	152
FIG. 3	Effect of carvacrol (CR) on germination and outgrowth of <i>C. difficile</i> BAA 1870 (3A) and 1805 (3B) spores.	153
FIG. 4	Effect of carvacrol (CR) on <i>C. difficile</i> ATCC BAA 1870 (4A) and 1805 (4B) toxin regulatory genes	155

Chapter VI

FIG. 1	Effect of CR supplementation on the incidence of <i>C. difficile</i> associated diarrhea in mice	186
FIG. 2	Effect of CR supplementation on the severity of CD associated disease in mice	188
FIG. 3	Effect of CR supplementation on relative weight loss in <i>C. difficile</i> infected and non-infected mice	190
FIG. 4	Effect of CR supplementation on the abundance of major gut microbiota (phyla level) in the antibiotic treated and <i>C. difficile</i> challenged mice	192
FIG. 5	Effect of CR supplementation on the abundance of Enterobacteriaceae (5a), Lactobacillaceae (5b) and Lachnospiraceae (5b) in the antibiotic treated and <i>C. difficile</i> challenged mice	194
FIG. 6	Effect of CR supplementation on the diversity of gut microbiota of antibiotic treated and <i>C. difficile</i> challenged mice	198
FIG. 7	Effect of CR supplementation on the diversity of gut microbiota of antibiotic treated and <i>C. difficile</i> challenged mice	200

List of Tables

Chapter III

TABLE 1.	MIC values of ten antimicrobials against the pork isolate of <i>C. difficile</i>	97
TABLE 2.	Antibiotic resistance genes identified in the genome of pork isolate SG-12 and multidrug resistant <i>C. difficile</i> 630	97

Chapter VI

TABLE 1.	Different treatment groups used in the experiment	183
TABLE 2a.	Mouse clinical score sheet	184
TABLE 2b	Mouse body condition chart	185

List of Abbreviations

CD	<i>Clostridium difficile</i>
CDAD	<i>Clostridium difficile</i> Associated Disease
CDC	Centers for Disease Control and Prevention
CDI	<i>Clostridium difficile</i> infection
CDMN	<i>Clostridium difficile</i> Moxalactam Norfloxacin medium
CFU	Colony forming Units
CR	Carvacrol
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
RT-qPCR	Real-time quantitative polymerase chain reaction
SAS	Statistical analysis software
SIC	Sub-inhibitory concentration
TC	<i>Trans</i> -cinnamaldehyde
USDA	United States Department of Agriculture
WHO	World Health Organization

Chapter I

Introduction

Clostridium difficile is a Gram-positive, spore-forming, anaerobic bacterium that causes a toxin-mediated enteric disease in humans (Weese, 2010). More than 300,000 cases of *C. difficile*-associated disease (CDAD) are reported annually in the United States, resulting in approximately US\$3 billion as healthcare costs (Ghose et al., 2007; Wilkins & Lyster, 2003). According to a study by the US Department of Health and Human Services, the New England region, including Connecticut reported the highest rate of *C. difficile* associated hospitalizations in 2010 and 2011 (Steiner et al., 2012). The emergence of a hypervirulent strain, NAP1/ribotype 027 that produces increased levels of toxins and a severe form of the disease in humans has been reported in the US (Blossom & McDonald, 2007; Hookman & Barkin, 2009; Sunenshine & McDonald, 2006). *C. difficile* predominantly affects long-term hospital inpatients and the elderly undergoing prolonged antibiotic therapy (Hookman & Barkin, 2009). Prolonged antibiotic therapy results in the disruption of the normal enteric microflora, leading to the germination of *C. difficile* spores and pathogen colonization in the intestine with subsequent production of toxins (Dial et al., 2005). *C. difficile* toxins, TcdA, and TcdB, act as glucosyl transferases that inactivate the Rho family GTPases associated with F-actin regulation and consequently cause disruption of the cytoskeleton and intestinal epithelial tight junctions (Keel & Songer, 2006; von Eichel-Streiber et al., 1999). This leads to an inflammatory response with the release of cytokines and leukotrienes, causing pseudomembrane formation in the intestine and watery diarrhea (Hookman & Barkin, 2009; McDonald et al., 2006; Sunenshine & McDonald, 2006). The genes *tcdA* and *tcdB*, which encode the toxins TcdA and TcdB, respectively, along with the genes encoding TcdR, an RNA polymerase sigma factor required for maximal expression of *tcdA* and *tcdB*, TcdC, an antagonist of TcdR, and TcdE, a holin-like protein needed for toxin excretion, collectively constitute the “pathogenicity

locus” in *C. difficile* (McDonald et al., 2006). Recent studies have suggested the possible emergence of new virulent or antibiotic resistant *C. difficile* strains by horizontal gene transfer between toxigenic and non-toxigenic strains (Brouwer et al., 2013). The Centers for Disease Control and Prevention (CDC) recently listed *C. difficile* as one among the three urgent threats in their report on emerging pathogens with antibiotic resistance (Steiner et al., 2012).

C. difficile disease has been traditionally regarded as a nosocomial infection in humans, especially in those receiving prolonged antimicrobial therapy (Bartlett, 1997; Hookman & Barkin, 2009; McDonald et al., 2006). However, in recent years, an increase in the number and severity of *C. difficile* infections in humans has been reported, particularly in those involving communities outside the hospital environment and in low-risk subjects (McDonald et al., 2006; Rupnik et al., 2009). Recent studies conducted by investigators worldwide have indicated the occurrence of *C. difficile* in a variety of food animals. A rise in the isolation rates of the pathogen from animal reservoirs is one potential reason attributed to the increased reports of human *C. difficile* infections (Rupnik et al., 2009; Thitaram et al., 2011). In addition, *C. difficile* has been isolated from raw and ready-to-eat meats at retail stores (Harvey et al., 2011a; Songer et al., 2009; Weese et al., 2009; Weese et al., 2010). The detection of genotypically similar and identical *C. difficile* strains implicated in human infections from foods and food animals (Rodriguez-Palacios et al., 2011; Weese et al., 2011) further highlights the potential role of food as a source of community-associated *C. difficile* disease (Marsh et al., 2011; Songer et al., 2009). Although a limited number of studies in the U.S have investigated the prevalence of *C. difficile* on different meats, an in-depth characterization of the meat isolates has not been performed. The prevalence of *C. difficile* on foods observed in previous investigations varies from 0 to 40% (Harvey et al., 2011b; Limbago et al., 2012; Songer et al., 2009; Weese et al., 2009).

Although exposure to broad-spectrum antibiotics predisposes patients to CDAD by disrupting the normal gut flora (Bartlett, 1992; O'Connor et al., 2004), antibiotics are still the primary line of treatment against *C. difficile*. In addition, the emergence of antibiotic resistance in hypervirulent strains of *C. difficile* is increasingly reported worldwide, which further limits the success of antibiotic treatment (Prabaker & Weinstein, 2011; Spigaglia et al., 2011). Since *C. difficile* toxins are the major virulence factors responsible for the pathogenesis of CDAD, identification of alternative therapeutic agents that inhibit *C. difficile* toxin production without disrupting the normal gastrointestinal flora would potentially constitute a viable approach for controlling CDAD.

Historically, plant compounds have been used for treating various diseases in traditional medicine. Carvacrol (CR) is a monoterpenoid phenol present in oregano and thyme oils. Diverse pharmacological actions of CR, including antimicrobial and anti-inflammatory properties, have been previously demonstrated (Baser, 2008). *Trans*-cinnamaldehyde (TC) is an aromatic aldehyde present as a major component of the bark extract of cinnamon. Both CR and TC are generally recognized as safe chemicals for use in foods (GRAS) by the U.S. Food and Drug Administration (FDA). Previous research conducted in our laboratory revealed that sub-inhibitory concentrations (SICs; the concentrations that do not inhibit bacterial growth) of TC and CR increased the sensitivity of multi-drug resistant *Salmonella* Typhimurium DT 104 to antibiotics by down-regulating antibiotic resistance genes and the efflux pump, *tolC* (Johny et al., 2010). In addition, our laboratory previously observed that TC inhibited biofilm synthesis and virulence in uropathogenic *Escherichia coli* (Amalaradjou et al., 2010; Amalaradjou et al., 2011).

Based on published literature and preliminary research conducted in our laboratory, it is hypothesized that meat is a potential source of multi-drug resistant *C. difficile*. Moreover, it was

hypothesized that CR and TC reduce *C. difficile* virulence by reducing toxin production and sporulation without deleteriously affecting the normal enteric flora. The specific objectives of this dissertation include

1. To determine the prevalence of *C. difficile* in retail meat sold in Connecticut, and to characterize *C. difficile* meat isolates by phenotypic and genotypic tests.
2. To investigate the effect of CR and TC on *C. difficile* toxin production *in vitro*.
3. To investigate the effect of CR on *C. difficile* sporulation and spore germination *in vitro*.
4. To study the effect of CR on *C. difficile* pathogenicity in a mouse model.

Chapter II

Review of Literature

C. difficile is a gram-positive, spore-forming, anaerobic bacterium, which causes a toxin-mediated enteric disease in humans (Hookman & Barkin, 2009). The symptoms of *C. difficile* disease include abdominal pain, fever, fulminant colitis, toxic megacolon (bowel perforation), sepsis and shock (Rupnik et al., 2009). In addition, asymptomatic colonization of *C. difficile* or mild diarrhea has also been reported in some patients (Hensgens et al., 2012). *C. difficile* infection has been associated with the use of antibiotics, gastric acid suppressing agents and resultant gut dysbiosis, which lead to the germination of spores in the stomach and selection for *C. difficile* in the intestine (Bartlett, 1992; Dial et al., 2005; Kelly & LaMont, 1998).

Recent evidence indicates that *C. difficile* has emerged as a community-associated pathogen (Beaugerie et al., 2003; Hensgens et al., 2012). *C. difficile* has been isolated from the intestinal tract of many food animals, and several small-scale studies conducted in different parts of the world have revealed the presence of *C. difficile* in retail meat and meat products (Rodriguez-Palacios et al., 2013a). These results raise concerns that foods could potentially be involved in the transmission of *C. difficile* to humans.

1. Epidemiology of *C. difficile* infection:

The total annual number of cases of *C. difficile* infection in the U.S. is estimated to exceed 500,000 (Lessa et al., 2015), resulting in approximately US\$3 billion annually as health care costs (O'Brien et al., 2007). *C. difficile* infections have been reported by physicians as increasing in severity and more difficult to treat than in the past (Hookman & Barkin, 2007; Sunenshine & McDonald, 2006). Among patients diagnosed with *C. difficile* infection, relapse or re-infection occurs in 12% to 24% within 2 months (Sunenshine & McDonald, 2006). Moreover, the mortality of the disease associated with *C. difficile* in the U.S. have increased from 5.7 per million to 23.7 per million from 1999 to 2004 (Redelings et al., 2007), and the incidence rate among hospitalized

patients doubled from 2001 to 2010 (Reveles et al., 2014).

Traditionally, *C. difficile* was considered a nosocomial pathogen that mainly affected the elderly, the severely ill, and long-term hospital inpatients (Hookman & Barkin, 2007; Hookman & Barkin, 2009). However, recently some changes in the epidemiology of *C. difficile* have been reported. For example, an increase in community-acquired *C. difficile* associated disease (CDAD) has been reported, especially in populations that were previously considered at low risk of the infection (Beaugerie et al., 2003; Lessa, 2013; Rupnik et al., 2009). In a case-control study on community acquired *C. difficile* infections, only half of the cases included in the study had received antibiotic therapy within four weeks of infection, and one-third had not been exposed to antibiotics or recently hospitalized (Wilcox et al., 2008). Another change in the epidemiology of *C. difficile* is that an increase in the morbidity, mortality and relapse rate in infections has been reported in the United States and elsewhere, which is attributed to the emergence and dissemination of a new hypervirulent strain, classified as North American Pulsotype 1 (NAP 1) by pulsed field gel electrophoresis (Arroyo et al., 2005; Rupnik et al., 2009). This strain belongs to the toxin type III and ribotype 027 (Loo et al., 2005). Emerging antimicrobial resistance in *C. difficile* has also been reported by many investigators, especially resistance to fluoroquinolones (Drudy et al., 2007; McDonald et al., 2005; McDonald, 2005; Sebahia et al., 2006), clindamycin and erythromycin (Qiong & Haihui, 2015), metronidazole (Musher et al., 2005; Pepin et al., 2005) and vancomycin (Kelly & LaMont, 2008). Moreover, a high-level resistance to gatifloxacin and moxifloxacin was reported in the NAP1/R 027 isolates (Kelly & LaMont, 2008; McDonald et al., 2005). In light of this, the CDC has listed *C. difficile* as one of the three urgent threats in their recent report on emerging pathogens with antibiotic resistance (Steiner et al., 2012)

2. *C. difficile* infection and pathogenesis

2.1. *C. difficile* associated disease (CDAD) in humans

C. difficile associated disease (CDAD) manifests as mild, moderate or severe disease in humans (Hookman & Barkin, 2009). The mild form of the disease is usually asymptomatic or manifested with mild fever and abdominal pain. Watery diarrhea is the principal symptom of moderate and severe CDAD in affected patients (Kelly & LaMont, 1998). In addition to watery and rarely bloody diarrhea, abdominal distension and leukocytosis are also seen in severe disease, which leads to complications and potentially death (Hookman & Barkin, 2009; Kelly & LaMont, 1998; Knight & Surawicz, 2013). Patients with severe CDAD may lose serum protein in stool leading to hypoalbuminemia, which in turn results in edema and occasional ascites (Dansinger et al., 1996; Rybolt et al., 1989). High fever, leukocytosis, and detectable toxins in the stool are of significant diagnostic value in clinical CDAD (Knight & Surawicz, 2013). Occasionally, patients suffer from abdominal pain caused by colonic distention and toxic megacolon. In addition, marked leukocytosis ($> 50,000/\mu\text{L}$) and lactic acidosis are indications of a serious clinical condition, which warrants surgical or advanced medical intervention (Knight & Surawicz, 2013).

2.2 Pseudomembranous Colitis

The classical sign of complicated CDAD is pseudomembranous colitis (Hookman & Barkin, 2009). Occasionally, other complications such as colonic perforation and sepsis may follow pseudomembranous colitis (Kelly & LaMont, 1998; Knight & Surawicz, 2013; Rupnik et al., 2009). Pseudomembranous colitis could be visualized using lower endoscopy as yellow membranous plaques in the colonic lumen formed by necrotic epithelial debris, inflammatory cells, and proteinaceous exudates (Hookman & Barkin, 2009). In advanced stages, pseudomembranous

colitis leads to sepsis and death in some patients, especially in the elderly patients (Knight & Surawicz, 2013).

2.3. Pathogenesis *C. difficile* associated disease (CDAD)

The mode of transmission of *C. difficile* infection in humans is primarily via the fecal-oral route (Hookman & Barkin, 2009). *C. difficile* spores are extremely resistant to physical and chemical agents, and can survive in the environment such as hospital surfaces and equipment for long periods (Bettin et al., 1994; Jabbar et al., 2010; Siani et al., 2011). Ingested *C. difficile* spores can survive the acidity of the stomach and reach the intestine, where, especially in the absence of normal gut microflora, spores germinate to vegetative cells in presence of a germinant, mainly taurocholic acid (Kelly & LaMont, 1998; Knight & Surawicz, 2013). Bile salts including taurocholic acid are actively secreted into the small intestine from the gall bladder, and most of the secreted bile salts are reabsorbed in the distal ileum. The residual bile salts remaining in the distal part of the intestine are readily converted to secondary bile salt metabolites by normal benign gut flora, which in turn minimize the availability of taurocholic acid for *C. difficile* spore germination (Kelly & LaMont, 1998; Knight & Surawicz, 2013). Therefore disruption of normal gut flora makes taurocholic acid available for *C. difficile* spore germination in presence of other co-germinants such as glycine. The disruption of normal gut flora is predominantly associated with long-term antibiotic therapy, and un-metabolized taurocholic acid serves as a potent germinant for *C. difficile* spores in clinical cases (McFarland, 2008; Wheeldon et al., 2011). The vegetative cells of the bacterium multiply in the intestinal crypts, producing toxin A and B, the major virulence factors of *C. difficile* (Kuehne et al., 2011).

C. difficile toxins inactivate Rho GTPases in colonic epithelial cells by glycosylation (Deng & Barbieri, 2008; Kuehne et al., 2011). Rho GTPases are critical in cell to cell adhesion,

cytokinesis, secretion, and most importantly in the maintenance of the cytoskeleton. The inactivation of GTPases by toxin A and B leads to the disruption of F-actin regulation, which in turn results in the disruption of the cytoskeleton and the intestinal epithelial cell tight junctions (Jafari et al., 2010; Rupnik et al., 2009). The disruption of epithelial tight junctions and increased epithelial permeability subsequently result in severe diarrhea. In addition, toxin A causes cell rounding, detachment, apoptosis, and induce the secretion of proinflammatory cytokines, including IL-8, a strong chemoattractant for neutrophils (El Feghaly et al., 2015; Monaghan, 2015; Solomon, 2013). Further, neutrophils cause severe tissue damage due to the release of lytic enzymes and free radicals. Moreover, toxin A interacts with macrophages in the lamina propria and causes further release of cytokines such as IL-12, IL-18, interferon gamma, IL-1 β , tumor necrosis factor alpha and other proinflammatory proteins (El Feghaly et al., 2015; Monaghan, 2015; Solomon, 2013). Altogether, necrotic epithelial debris admixed with inflammatory cells and exudates form a pseudomembrane over the colonic luminal surface which is presented as a classical pseudomembranous colitis (Hookman & Barkin, 2009). In adverse conditions, exuberant inflammatory reactions and release of systemically active cytokines, complicated by fluid loss resulting from diarrhea may culminate in systemic shock and death (Knight & Surawicz, 2013).

2.4 *C. difficile* toxins

Similar to several other bacterial exotoxins, *C. difficile* toxins are high molecular weight proteins (Popoff & Bouvet, 2009; Voth & Ballard, 2005). *C. difficile* toxin A (TcdA, enterotoxin) and toxin B (TcdB, cytotoxin) which are responsible CDAD in humans represent the major virulence factors of the pathogen (Kelly & LaMont, 1998; Stanley et al., 2013). These toxins are members of a family of large clostridial toxins (LCTs), produced by pathogenic Clostridia such as *C. sordellii*, *C. novyi*, *C. perfringens* (Davies et al., 2011; Popoff & Bouvet, 2009). In addition,

binary toxins (CdtA and CdtB) are produced from various hypervirulent *C. difficile* strains can also contribute to the severity of CDAD in affected patients. (Bacci et al., 2011; Barth & Stiles, 2008; Gerding et al., 2014).

C. difficile toxins, TcdA and TcdB are 308 and 270 kDa proteins, respectively, with 49% identity and 63% similarity in their amino acid sequence (Davies et al., 2011). Both TcdA and TcdB are homologous AB toxins; A subunit with catalytic activity and B subunit associated with the delivery of A subunit into host cells (Davies et al., 2011; Popoff & Bouvet, 2009). The A subunit is structurally an N-terminal glucosyltransferase (GTD) domain that inactivates Rho family GTPases in the host cell (Voth & Ballard, 2005). Three additional domains present in the B subunit associated with the delivery of GTD into the target cell include receptor-binding domain, pore-forming domain, and autoprotease domain. These combined AB domains in *C. difficile* toxins act in four steps 1) binding and internalization, 2) pore-formation and translocation of the GTD, 3) autoproteolysis and release of the GTD 4) inactivation of host GTPases by glycosylation (Pruitt & Lacy, 2012).

Both TcdA and TcdB induce rounding of affected cells (Pruitt & Lacy, 2012). Despite a comparable structure and mechanism of action, TcdA and TcdB have differences in their phenotypic characteristics. It has been demonstrated that TcdB is 100–10,000 times more potent than TcdA in its cytopathic effect in various cell types (Voth & Ballard, 2005). In addition to the cell rounding, TcdA and TcdB can cause cells death (cytotoxic effect) through apoptosis and necrosis (Chumbler et al., 2012; Kuehne et al., 2011). Earlier studies suggested TcdA as a key virulence factor in CDAD, where it causes initial damage in the enterocytes, which facilitate the entry and further damage by TcdB (Gerhard et al., 2008; Kuehne et al., 2011; Sutton et al., 2008). However, recent studies demonstrated TcdB as the major virulence factor in CDAD, and TcdA is

not essential for CDAD pathogenesis in humans (Lyras et al., 2009; McDonald et al., 2005). Moreover, TcdA⁻TcdB⁺ strains of *C. difficile* cause the similar degree of damage as TcdA⁺TcdB⁺ strains in humans, and no pathogenic TcdA⁺TcdB⁻ strains have been detected in humans (Sambol et al., 2000).

Similar to TcdA and TcdB, the binary toxins also act as ADP-specific ribosyltransferase, which also disrupt actin (Barth & Stiles, 2008; Gerding et al., 2014). The binary toxins augment the adherence of *C. difficile* to the target cells and contribute to virulence in *C. difficile* infections in humans (Gerding et al., 2014; Hemmasi et al., 2015; Schwan et al., 2009). Interestingly, it was observed that TcdA⁻TcdB⁻, binary toxin-producing strains of *C. difficile* can cause enterocyte damage, indicating the significance of binary toxins in CDAD pathogenesis (Gerding et al., 2014).

2.5. Regulation of *C. difficile* toxin production

C. difficile toxins TcdA and TcdB are encoded by *tcdA* and *tcdB*, respectively located in a single operon, and expressed during the late log phase and early stationary phases of bacterial growth (Voth & Ballard, 2005). TcdA and TcdB, associated regulatory proteins TcdR and TcdC along with holin-like protein, TcdE are encoded in a 19.6 kb genomic region known as the Pathogenesis locus (Paloc) of the *C. difficile* genome (Dingle et al., 2013; Neyrolles et al., 2011). TcdR, the positive regulator of *C. difficile* toxin production, is functionally an alternative sigma factor that promotes TcdA and TcdB transcription (Bouvet & Popoff, 2008; Dupuy & Matamouros, 2006). TcdC is a negative regulator of toxin production within the Paloc region, which inhibits TcdR-dependent toxin gene transcription binding to TcdR complex (Dupuy et al., 2008; Matamouros et al., 2007). In addition, TcdE, a porin protein helps in the delivery of *C. difficile* toxins to the exterior of the bacterial cell (Govind & Dupuy, 2012; Govind et al., 2015).

In addition to the regulatory genes located in the Paloc region, *C. difficile* toxin production

is regulated by genes located outside the pathogenicity locus, such as *codY* and *ccpA* (Antunes et al., 2011a; Dineen et al., 2010). The CodY is a global regulator encoded outside of the PaLoc region, which functions as a strong repressor of toxin production (Dineen et al., 2007). CodY is a global repressor of virulence associated genes in various Gram-positive bacteria, including clostridia (Dineen et al., 2007; Fisher et al., 1996; Guedon et al., 2001; Lobel et al., 2012). In *C. difficile*, CodY is expressed in response to branched chain amino acids, binds to the *tcdR* promoter region, which in turn suppresses toxin production (Dineen et al., 2007).

In addition to CodY, another important regulatory system for *C. difficile* toxin production is CcpA mediated repression (Antunes et al., 2011b). CcpA, a member of the LacI/GalR family of transcriptional regulators, takes part in carbon catabolite repression in low G+C Gram-positive bacteria (Antunes et al., 2011b; Antunes et al., 2012; Li et al., 2015). Carbon catabolite repression system recognizes rapidly metabolizable carbon sources such as glucose in the medium and acts upon several genes responsible for nitrogen and carbon metabolism (Antunes et al., 2011b). Moreover, in several pathogenic Gram-positive bacteria such as *Clostridium* and *Bacillus*, CcpA regulates virulence-associated genes (Jiang et al., 2015; Li et al., 2015). Similarly, in *C. difficile*, CcpA recognizes glucose and other carbon sources in the media and suppresses the toxin production, sensing a nutritious environment (Antunes et al., 2011b). In addition, it has been demonstrated that sporulation gene, *spo0A* regulates both sporulation and toxin production in *C. difficile* (Pettit et al., 2014). However, the mechanism is yet to be delineated.

3. *C. difficile* sporulation

3.1 *C. difficile* spores

Sporulation is a critical virulence factor in *C. difficile* pathogenesis. *C. difficile* spores being survival structures that can withstand unfavorable physical, chemical and metabolic conditions,

play an important role in *C. difficile* persistence in the environment, transmission to new susceptible individuals and relapse in temporarily recovered patients (Paredes-Sabja et al., 2014c). Sporulation in *C. difficile* is a complex and less understood process. Vegetative cells of *C. difficile* readily sporulate during egestion, contaminate the environment and initiate a new infectious cycle through feco-oral route. In laboratory conditions, *C. difficile* sporulates in early stationary phase or death phase; however, the cues that initiate *C. difficile* sporulation are not fully identified. Various environmental stimuli such as scarcity of nutrients, physical and chemical stress factors, oxidative stress (aerobic environment) and quorum sensing are considered as initiators of *C. difficile* sporulation (Higgins & Dworkin, 2012; Paredes-Sabja et al., 2014c).

Sporulation process in *C. difficile* involves an asymmetric division of sporulating vegetative cells to a forespore and a mother spore. The formation of and the interaction between these two compartments yield highly resistant and metabolically dormant survival structures (Burns & Minton, 2011; Edwards & McBride, 2014; Talukdar et al., 2014). The larger mother spore engulfs the smaller forespore, which eventually becomes the functional spore. *C. difficile* spores are composed of seven distinct layers or compartments. The innermost compartment is the spore core, which carries the genetic elements and essential proteins, including enzymes (Paredes-Sabja et al., 2014c). Low water content and high dipicolinic acid concentration in the spore core are predominantly responsible for spore resistance reported in *C. difficile* (Setlow, 2007). Enveloping the spore core is the inner membrane, which is mainly composed of phospholipids (Cowan et al., 2004). The inner membrane is surrounded by germ cell wall, which is further protected by a thick peptidoglycan layer, which in turn is covered by an outer membrane formed from the mother cell. The entire structure is further surrounded by a proteinaceous spore coat, which provides resistance to most disinfectants.

3.2 Regulation of *C. difficile* sporulation

The sporulation process in *C. difficile* is regulated by the master regulator *spoOA*. (Saujet et al., 2014). Loss of function mutation of *spoOA* has been shown to considerably impair the transmission of *C. difficile* infection in mice (Deakin et al., 2012a). Phosphorylation of Spo0A is an essential step in the initiation of the sporulation process, where several histidine kinases, including s CD1352, CD1492, CD1579, CD1949, and CD2492, encoded by *cody* play a vital role. *C. difficile* orphan histidine kinase CD1579 autophosphorylate and transfer a phosphate group to Spo0A (Underwood et al., 2009). However, the role of other histidine kinases is yet to be known. The regulation of sporulation in *C. difficile* is a complex process chiefly controlled by the RNA polymerase sigma factors (σ^F , σ^E , σ^G , and σ^K). Sigma factors σ^F and σ^G are associated with forespores, and σ^E and σ^K are associated with the mother cell (Fimlaid et al., 2013). The forespore protein SpoIIR is needed for σ^E activation (Saujet et al., 2014), which is dependent on SpoOA, the master regulator. *spoIIID* encodes for a regulator in the mother cell. The sigma factors σ^E and σ^G and *spoIIID* are critical for σ^K production and activation (Paredes-Sabja et al., 2014a). In addition, SpoIVA is essential for the synthesis of spore coat surrounding the forespore (Putnam et al., 2013).

3.3 *C. difficile* spore germination

Patients contract *C. difficile* infection by ingestion of spores. Germination of spores in the small intestine to form vegetative cells is an important step in initiating CDAD (Paredes-Sabja et al., 2014b). Generally, germination of Clostridial spores is induced by specific germinant receptors that sense distinctive small molecules, which are highly species specific. Activation of these receptors induce the release of monovalent cations such as H^+ , Na^+ , and K^+ and dipicolinic acid chelated to calcium (Ca-DPA) stored in the spore core (Paredes-Sabja et al., 2011). Release Ca-DPA activates cortex hydrolases that degrade the peptidoglycan layer in the cortex, subsequently

leading to hydration of the spore core and reactivation of the spore core metabolism (Paredes-Sabja et al., 2011). In closely related species like *C. perfringens*, induction of the cortex hydrolase protein SleC, depends on its cleavage by another family of proteases, including CspA, CspB, and CspC (Labbe et al., 1978; Paredes-Sabja et al., 2011). In *C. difficile* also, CspC is established as a spore germinant receptor (Burns et al., 2010; Francis et al., 2013; Paredes-Sabja et al., 2014b). *C. difficile* spore germination is induced by specific bile salts, cholate and its derivatives (glycocholate, taurocholate, cholate and deoxycholate) in the presence of a cogerminant L-glycine. However, germination of *C. difficile* spores in the absence of these small molecules has been observed in experimental settings, suggesting that other complex pathways are involved in this process (Francis et al., 2013; Giel et al., 2010; Sorg & Sonenshein, 2008). It is demonstrated that CspC triggers Ca-DPA release from the spore core upon activation by taurocholate, however, the exact mechanism is not clear. In *C. difficile*, the serine protease, CspB is required for the conversion of pro-SleC into mature SleC resulting in cortex hydrolysis and spore germination (Francis et al., 2013; Paredes-Sabja et al., 2014b).

4. *C. difficile* and gut microbiome

A healthy and normal gut microflora is crucial for preventing pathogen colonization, including *C. difficile* (Britton & Young, 2014). Therefore, the most important predisposing factor for *C. difficile* infection is the disruption of normal gut microbiota (Hookman & Barkin, 2009). Antibiotic therapy significantly alters the microbial diversity; and in many case changes in the microbial diversity persist even after withdrawing the antibiotic (Antonopoulos et al., 2009; Dethlefsen et al., 2008b). The major antibiotic or antimicrobial classes that are documented to predispose *C. difficile* infection include fluoroquinolones clindamycin, cephalosporins, and penicillins (Blossom & McDonald, 2007; McFarland, 2008).

Throughout life, the human gut microbiota undergoes continuous shifts and alterations (Hopkins et al., 2001a). The gut microbiome of a healthy adult is almost stable; however, the microbial composition undergoes significant alterations and becomes less diverse as age advances (Biagi et al., 2010; Claesson et al., 2011; Hopkins et al., 2001a). In elderly people, protective populations such as *Bifidobacterium* and other members of Firmicutes considerably diminish accompanied by an increase in undesirable species of *Proteobacteria* in the gut (Biagi et al., 2010; Claesson et al., 2011; Hopkins et al., 2001b). Moreover, age-related senescence in the immune status of the elderly, along with frequent hospital visits during old age contribute to a detrimental alteration in the gut microbiome and subsequent colonization of *C. difficile* (Seekatz & Young, 2014).

Other important factors that detrimentally affect gut microbiota and predispose *C. difficile* infection are the use of proton pump inhibitors and chronic gastrointestinal diseases (Berg et al., 2013; Dial et al., 2005; Vesper et al., 2009). Proton pump inhibitors alter the pH of the gut, thereby affecting microbial populations, especially beneficial bacteria such as *Lactobacillus* species (Altman et al., 2008; Vesper et al., 2009). In addition, conditions such as inflammatory bowel disease induce significant gut dysbiosis, reducing the diversity of Firmicutes and Bacteroides population, accompanied by an increased Proteobacteria in the gut of affected patients (Manichanh et al., 2006; Nagalingam & Lynch, 2012).

The Firmicutes to Bacteroides ratio in gut increases as age advances and an elevated ratio is an indication of gut dysbiosis in humans (Ling et al., 2014; Mariat et al., 2009). Similarly, in *C. difficile* infected patients, a significant increase in Firmicutes to Bacteroides ratio is observed (Ling et al., 2014). Moreover, a decrease in Firmicutes, especially the depletion of *Ruminococcaceae*, *Lachnospiraceae*, and butyrogenic bacteria within this phylum has been found to be associated

with *C. difficile* infection and nosocomial diarrhea in humans (Antharam et al., 2013). In addition, *C. difficile* infected patients were reported to possess a higher gut count of Enterobacteriaceae (Proteobacteria) and decreased Enterococcaceae (Firmicutes) (Hopkins & Macfarlane, 2002; Rea et al., 2012; Schubert et al., 2014).

Interestingly, a majority of infants are colonized with *C. difficile*, however; they do not develop the disease (Rousseau et al., 2011). It is found that a comparatively higher population of *Bifidobacteria* in the gut exerts a protective effect against *C. difficile* in infants (Rousseau et al., 2011). Moreover, healthy young individuals have a higher population of *Bifidobacteria* and more diverse *Bacteroides* compared with elderly people (Rea et al., 2012). Altogether *C. difficile* infected patients possess a less diverse gut microbiota compared to non-infected healthy adults (Rea et al., 2012).

5. Treatment strategies of *C. difficile* infection in humans

Medical management of *C. difficile* infection is still one of the most problematic therapeutic challenges in hospital settings. The treatment of *C. difficile* infection is often complicated by relapse of infection that ranges from 20% after the first episode to 60% after multiple events of recurrence. The treatment strategies against CDAD is multifactorial and involve diverse approaches to address bacterial colonization of the gut, toxin-mediated colonic inflammation, fluid loss due to diarrhea, disruption of the gut microbiome, and transmission and relapse of infection due to *C. difficile* spores.

5.1 Antibiotic therapy

Antibiotic treatment against CDAD is chosen based on the severity and recurrence rate of the infection. Metronidazole or vancomycin treatment for 10 to 14 days has been found to be effective in 50% of the patients after the first episode of a recurrent *C. difficile* infection (Longo et

al., 2015). Subsequent episodes of recurrent *C. difficile* infections are difficult to treat due to the persistence of spores in the gut and an inadequate immunologic response against *C. difficile* toxins. Recurrent *C. difficile* infection is usually managed with a decreasing or pulsed regimen of vancomycin. A pulsed regimen of vancomycin is reported to be more effective against recurrent CDI compared to conventional dosing (McFarland et al., 2002). A recently developed antibiotic, namely fidaxomicin has been found to be more efficacious in treating CDAD after the second and subsequent episodes of CDAD (Zhanel et al., 2015). Moreover, fidaxomicin was found to be more effective than vancomycin in preventing relapse and re-infection in affected patients (Housman et al., 2015; Zhanel et al., 2015). Fidaxomicin was also shown to reduce *C. difficile* toxin production and sporulation *in vitro*, suggesting an improved clinical outcome and reduced incidence of relapse in treated patients (Allen et al., 2013; Babakhani et al., 2013; Babakhani et al., 2012). Other anti-*C. difficile* antibiotics include rifaximin, nitazoxanide, teicoplanin, ramoplanin, and tigecycline; however, their therapeutic use is limited in practical settings due to a lack of adequate clinical data, antibiotic resistance potential, higher cost of treatment.

The efficacy of antibiotics, including fidaxomicin is limited in patients suffering from severe colitis associated with advanced *C. difficile* infection. In such cases, emergency colectomy or a diverting ileostomy with a colonic lavage with vancomycin is usually performed. Moreover, *C. difficile* has been reported to acquire resistance against most of the currently used antibiotics (Blossom & McDonald, 2007; Prabaker & Weinstein, 2011; Qiong & Haihui, 2015; Spigaglia et al., 2011; Spigaglia et al., 2008; Steiner et al., 2012), thereby warranting alternate and effective therapeutic strategies that can control the pathogen.

5.2 Fecal microbial transplantation

The colonic microflora provides colonization resistance against most of the microbial

pathogens, and its disruption due to antibiotic therapy is the major predisposing factor of CDAD. Oral antibiotic therapy results in a rapid decline in colonic microbial diversity and this change can last several weeks to months. Terminating the antibiotic regimen is critical in eliminating *C. difficile* from the intestine and initiating the recovery of microbial composition and diversity (Ofosu, 2016a). However, a complete replenishment of the colonic microbiome may take several weeks, and the relapse of *C. difficile* infection can occur within this period (Dethlefsen et al., 2008a; Jernberg et al., 2007). Fecal microbial transplantation has recently been introduced as a safe and effective treatment against recurrent *C. difficile* infection. Fecal microbial transplantation is documented to prevent relapse of CDAD in patients with a history of recurrent infection (van Nood et al., 2013). This procedure involves screening of healthy donors, the collection of fecal material that contains important bacteria particularly from the *Bacteroidetes* and *Firmicutes* phyla, and introducing the fecal material to the colon of the recipient orally or rectally (Ofosu, 2016b; Shahinas et al., 2012). This method was first recorded in 1958 and recently emerged as an effective strategy against CDAD with more than 90% success rate (EISEMAN et al., 1958; Kassam et al., 2013). Fecal transplantation along with parallel anti – *C. difficile* antibiotic therapy was also found to be more efficacious than administering antibiotics alone (van Nood et al., 2013). However, the exact mechanism of how fecal microbial transplantation prevents recurrent CDAD is not known. In addition, the ideal composition of specific bacterial taxa and their significance in conferring optimum colonization resistance against *C. difficile* in the colon is also unknown. Moreover, screening of healthy donors and ruling out the possibility of transmission of other infectious disease through transplanted fecal material are critical steps in this method (Ofosu, 2016b; Pacheco & Johnson, 2013). Fecal transplantation is still not considered as a primary line of treatment against CDAD, and further investigations on this approach are currently underway

(Ofosu, 2016a). The efficacy of administering capsules containing the spores of fecal microbial flora compared to the conventional fecal transplantation is also being investigated.

5.3 Vaccination

Animals immunized with toxoids prepared from TcdA and TcdB have been reported to possess resistance against *C. difficile* infection. Results revealed that specific anti-toxin serum IgG can successfully prevent toxin-mediated colitis in animals, suggesting its potential as a vaccination strategy against human CDAD (Ghose et al., 2007; Siddiqui et al., 2012). In addition, passive immunization with monoclonal anti-toxin immunoglobulin was found effective in animal models (Zhao et al., 2014). However, the cost effectiveness is a major concern in primary infections (Ofosu, 2016a; Solomon, 2013). The toxoid A and B parenteral vaccines have been found safe and relatively effective in human subjects (Kotloff et al., 2001). However, it should be noted that vaccination fails to prevent *C. difficile* colonization in gut, but it can only ameliorate the severity of infection by blocking toxin-mediated pathology (Longo et al., 2015).

5.4 Probiotics

Probiotics are live microorganism that when administered in adequate amounts confer a health benefit on the host (Sanders, 2008). Probiotics are widely considered to exert preventive and prophylactic potential against CDAD and its recurrence. Several theories have been postulated to explain the protective effect of probiotics against various enteric diseases, including CDAD. The protective effect of probiotics is primarily attributed to their role in inhibiting or modulating pro or anti-inflammatory signaling pathways in the gut (Kumari et al., 2011; Patel & Lin, 2010). However, in the case of CDAD, probiotics are prescribed as an adjunct therapy with antibiotics to maintain gut microflora, which is eliminated or disrupted during antibiotic treatment. Many clinical trials with probiotic bacteria such as *Saccharomyces boulardii*, *Lactobacillus casei* and *L.*

acidophilus have demonstrated their beneficial effect against recurrent CDAD (Johnson et al., 2012).

Various studies have reported beneficial effects of *S. boulardii* in preventing CDAD incidence in patients undergoing antibiotic therapy (Surawicz et al., 2000). Moreover, probiotic administration significantly reduced the recurrence of CDAD in infected patients. (Crow et al., 2015). In a controlled trial, the patients undergoing antibiotic therapy were provided with a cocktail of *Lactobacillus casei*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* or a placebo drink, and the incidence of CDAD was recorded in treatment groups. No CDAD was reported in probiotic cocktail group as opposed to 17% incidence in the placebo group (Hickson et al., 2007). *Lactobacillus plantarum* 299v administration has also been reported to prevent CDAD in hospitalized patients undergoing antibiotic therapy (Kujawa-Szewieczek et al., 2015). Although several studies are suggesting the beneficial effects of probiotic bacteria in preventing CDAD and its recurrence in patients, the use of probiotics is limited to a prophylactic approach, and further studies are required to confirm their effectiveness for treating CDAD.

5.5 Alternative therapeutic strategies against CDAD

All therapeutic strategies currently available to prevent CDAD have their limitations, and *C. difficile* is still considered as one of the most ‘difficult to tackle’ nosocomial pathogens, thereby triggering an intensive search for alternative approaches to prevent CDAD. Alternate therapeutic strategies under investigation to prevent *C. difficile* infection include the use of bacteriophages, non-toxigenic *C. difficile* strains, and anti-virulence small molecules. Bacteriophages have been approved for use to prevent foodborne pathogens (Hagens & Loessner, 2010). The critical part of developing bacteriophage therapy against a bacterial pathogen is identifying and screening of a specific phage that can selectively target the bacterium of interest. A specific four-phage

combination (phiCDHM1-phiCDHM2-phiCDHM5-phiCDHM6) has been documented to be effective in killing *C. difficile*(reference). Results from animal studies indicated administration of bacteriophage combinations significantly reduced *C. difficile* colonization in the gut (Nale et al., 2015). The major drawback in the use of bacteriophages is their fragile nuclear composition and possibility of transfer of virulence factors between bacterial pathogens. Interestingly, phage tail-like particles (PTLP) have been identified which can function similar to bacteriophages, but do not transfer their DNA to the target cell (Sangster et al., 2015). However, further investigations are required to confirm their practical use in human patients.

Another novel therapeutic approach against CDAD is the use of nontoxigenic *C. difficile* for facilitating competitive exclusion of pathogenic *C. difficile* in the gut. *C. difficile* strain VP20621 was found to be able to colonize the gastrointestinal tract of CDI infected patients (Villano et al., 2012). Moreover, nontoxigenic *C. difficile* strain M3 (VP20621; NTCD-M3) has been shown to reduce pathogenic *C. difficile* colonization when used with antibiotics such as vancomycin and metronidazole (Gerding et al., 2015).

Since the toxins constitute the major virulence factor that is directly associated with *C. difficile* associated colitis and clinical symptoms, a search for anti-virulence drugs that can reduce the production or activity of *C. difficile* toxins A and B is an emerging research topic. Targeting of the cysteine protease domain (CPD) located within the toxin protein has been found to be effective in neutralizing *C. difficile* toxin-mediated pathology. Cysteine protease domain inhibitors, such as ebselen, has been shown to possess activity against both TcdA, and TcdB and prevented CDAD in mice (Bender et al., 2015). The increased incidence and severity of CDAD along with high recurrence and relapse rates coupled with the emergence of antibiotic-resistant

strains have incited significant research interest in the discovery of anti-virulence drugs against *C. difficile*. However, only a few compounds have been identified so far in this category.

6. Plant-derived phenols

6.1 Carvacrol

Carvacrol (CR) is phytophenol present in oregano oil obtained from *Origanum vulgare*, a herb natively seen in Europe and the Mediterranean. Carvacrol is classified as Generally Recognized as Safe (GRAS) and approved for use in foods by the FDA. The essential oil extracted from *O. vulgare* has been found effective against various bacterial and fungal infections affecting different body systems (Nostro & Papalia, 2011). Oregano oil and the active component carvacrol have been found to possess significant antibacterial activity against various Gram-positive and Gram-negative bacteria, including *Helicobacter pylori*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, and *Streptococcus mutans*, *Enterobacter sakazakii*, *Haemophilus influenza* and methicillin-resistant *S. aureus* (Botelho et al., 2009; Chun et al., 2005; Hersch-Martinez et al., 2005). Moreover, CR has been found to exert inhibitory effect on spore outgrowth of *Clostridium perfringens* (Juneja & Friedman, 2007).

Besides the antibacterial property, CR was found to possess various pharmacologic actions, including anti-inflammatory, anti-diarrheal, anti-cancer, anti-adipogenic and neuroprotective effects (Baser, 2008; Hotta et al., 2010; Kim et al., 2013; Lima et al., 2013; Yu et al., 2012). Carvacrol was shown to exhibit strong anti-oxidant and anti-inflammatory properties by reducing the accumulation of free-radicals and reducing the synthesis of inflammatory mediators (Hotta et al., 2010). Carvacrol increased the anti-oxidant enzymes such as superoxide dismutase (SOD) and glutathione (GSH) levels and reduced myeloperoxidase (MPO) and nitric oxide (NO) along with inhibition of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), and

interleukin-1 beta (IL-1 β) (Arigesavan & Sudhandiran, 2015). Suppression of the synthesis of inflammatory mediators by CR is attributed to the inhibition of COX2 enzyme in macrophages (Hotta et al., 2010) and induction of anti-inflammatory cytokine IL-10 (Lima et al., 2013).

6.2 *Trans*- cinnamaldehyde

Trans-cinnamaldehyde (TC) is one of the principal components of the bark extract of cinnamon. It is also classified as GRAS by the FDA. Based on chronic toxicity studies, the U. S. Flavoring Extract Manufacturers' Association reported that TC possesses a wide margin of safety with no observed adverse effect levels (Adams et al., 2004). No identifiable genotoxic and mutagenic effects were reported in these studies. Studies conducted in rats revealed that oral supplementation of cinnamaldehyde resulted in 3.2 to 16% recovery of the compound in excreta, with 0.7% retention in the body tissues (Peters & Caldwell, 1994; Sapienza et al., 1993).

Trans-cinnamaldehyde exhibits antimicrobial activity against a wide range of pathogens, including Gram-positive and Gram-negative bacteria (Friedman et al., 2002). Antibacterial effect of TC against *Listeria monocytogenes*, *S. aureus*, *Vibrio spp.* and *E. coli* 0157: H7 has been documented by various researchers (Brackman et al., 2008; Moon & Rhee, 2016; Upadhyay et al., 2012; Upadhyay & Venkitanarayanan, 2016; Zhang et al., 2014). Interestingly, TC was found to inhibit various pathogenic bacteria *in vitro* without causing any deleterious effect on the normal gut microflora. *Trans*-Cinnamaldehyde was found to reduce the growth of *Salmonella* Typhimurium DT104 and *E. coli* 0157: H7 with minimal inhibitory effects on *Lactobacilli* and *Bifidobacteria* (Si et al., 2006). Moreover, TC has been found to be effective against biofilm formation in *Cronobacter sakazakii* and uropathogenic *E. coli* (Amalaradjou et al., 2009; Amalaradjou et al., 2010; Amalaradjou et al., 2011; Amalaradjou & Venkitanarayanan, 2011a; Amalaradjou & Venkitanarayanan, 2011b). Furthermore, experiments conducted in our laboratory

revealed that supplementation TC can reduce cecal colonization of *S. Enteritidis* in chickens and inhibit critical virulence factors associated with its egg-borne transmission (Kollanoor Johny et al., 2010; Kollanoor-Johny et al., 2012; Upadhyaya et al., 2013; Upadhyaya et al., 2015).

7. Potential food-borne transmission of *C. difficile*

7.1 Food animals as a source of *C. difficile*

Animals are an important source of human infectious microorganisms, especially those causing enteric disease, which can spread following direct or indirect contact, through environmental contamination or food (Steinmuller et al., 2006). For example, major foodborne pathogens such as *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* species are carried by food animals in their gastrointestinal tract, and are shed in feces (Altekruse et al., 1997), thereby contaminating the animals' hide, environment, water supply, and most importantly, the food supply. Studies conducted by many investigators worldwide have indicated the occurrence of *C. difficile* in a variety of food animals, and one of the reasons attributed to the increased reports of human *C. difficile* infections is the rise in the isolation rates of the pathogen from animal reservoirs (Indra et al., 2009; Rodriguez-Palacios et al., 2013a; Rupnik et al., 2008).

7.1.1 *C. difficile* infection and clinical signs in domestic animals

C. difficile associated diarrhea is reported in several animal species, which include pigs, cattle, horses, dogs, hamsters, guinea pigs, ostriches and elephants (Keessen et al., 2011a). *C. difficile* is a major cause of neonatal enteritis in pigs (Songer & Uzal, 2005). Affected piglets usually exhibit yellow watery diarrhea or constipation with varying degrees of colitis seen at autopsy (Songer & Anderson, 2006). *C. difficile* infection in foals and adult horses results in mild disease with diarrhea to fatal hemorrhagic necrotizing enterocolitis (Keessen et al., 2011a). *C. difficile* has been isolated from clinical cases of diarrhea reported in calves. However, a direct

correlation between diarrhea and *C. difficile* infection in calves, dogs, cats and poultry are poorly understood.

7.1.2 Prevalence of *C. difficile* in food animals

In a study involving 600 piglets with enteritis, 35% of the animals were found infected with *C. difficile* (Songer, 2004; Songer & Uzal, 2005). Another study examining piglets with enteritis reported the presence of *C. difficile* in more than one-third of the animals and concluded that *C. difficile* is potentially the most significant uncontrolled cause of neonatal diarrhea in pigs (Songer & Anderson, 2006). Similarly, studies conducted in different countries suggested a high prevalence of *C. difficile* in pigs, where the pathogen was genetically similar to some of the human clinical isolates (Pirs et al., 2008; Silva et al., 2015; Squire & Riley, 2013; Thitaram et al., 2011a; Thitaram et al., 2016; Yaeger et al., 2007). A study conducted in the US isolated *C. difficile* ribotype 078 from 83% of neonatal pig samples and 94% of calf samples (Keel et al., 2007). In the Netherlands, 078 *C. difficile* strains were detected from pigs and humans with comparable phenotypic and genotypic characters (Debast et al., 2009). *C. difficile* has been isolated more readily from young animals such as piglets and but adult animals prior to slaughter also tested positive for the pathogen (Costa et al., 2012; Rodriguez et al., 2012; Silva et al., 2015). A study conducted in the US with 875 food animals identified a comparatively lower prevalence rate of <0.6% in poultry, cattle and pigs (Rodriguez-Palacios et al., 2014). In separate studies conducted in different parts of the world, *C. difficile* has been detected in calves with varying prevalence rates ranging from 1.8% in Slovenia (Pirs et al., 2008) to as high as 49% in Canada (Costa et al., 2012; Weese, 2010). Furthermore, human hypervirulent *C. difficile* strain ribotype 027 has also been isolated from calves (Rodriguez-Palacios et al., 2006). In the United States, a prevalence rate of

25% in stool specimens collected from 253 diarrheic calves was reported, indicating that the bacterium is an important pathogen of young animals (Hammitt et al., 2008).

Besides clinically sick animals with *C. difficile*, an important and problematic public health significance is the shedding of the pathogen by clinically normal animals. These animals could potentially constitute a large and undetected reservoir of the pathogen for contamination of food, water and the environment (Weese, 2010). Researchers have established that calves can be asymptomatic carriers of toxigenic *C. difficile* and may serve as a significant source (Hammitt et al., 2008; Rodriguez-Palacios et al., 2013a). In study conducted in Austria, of the 59 fecal specimens collected from healthy broiler chickens slaughtered, 5% of the samples tested positive for *C. difficile* (Indra et al., 2009). Notably, 3.4% of these samples yielded toxigenic ribotypes 001 and 446, with the ribotype 001 being the second most frequently isolated strain implicated in humans *C. difficile* infections in that country (Indra et al., 2009). In the same study, *C. difficile* was isolated from 4.5 and 3.3% of the gut samples collected from adult cattle and pigs, respectively. A study conducted by the U.S. Department of Agriculture in Texas revealed 2% prevalence of *C. difficile* in commercial chickens prior to harvest (Harvey et al., 2011a). All these aforementioned studies collectively suggest food animals as a potential source of *C. difficile* relevant to public health.

7.2. Animal derived foods as a source of *C. difficile*

Since food animals are a potential source of *C. difficile* infection, animal-derived foods could be one of the transmission routes of *C. difficile* from animals to humans. Moreover, detection of genotypically similar and identical *C. difficile* strains implicated from human infections in food animals raised concerns on the role of food as a source of human infections. Validating these concerns, *C. difficile* spores have been isolated from a variety of raw and ready-to-eat meats

intended for human consumption. The first report on the contamination of retail meat with *C. difficile* was from Canada, where the bacterium was isolated from 21% of 53 ground beef and 14% of 7 ground veal samples collected from five stores in two Canadian provinces (Rodriguez-Palacios et al., 2007). The toxigenic *C. difficile* North American pulsotype NAP1, toxinotype III constituted 67% of the isolates from this study. A follow-up study by the same group of investigators involving three Canadian provinces over a period of eight months, reported an isolation rate of 6.7% and 4.6% in ground beef and veal chops, respectively (Rodriguez-Palacios et al., 2009). Further, a series of studies conducted in Canada also found that *C. difficile* was present in ground meats at the retail level, with a prevalence of 12% each in ground beef and ground pork and 12.8% in chicken (Weese et al., 2010a; Weese et al., 2009). These initial findings encouraged investigators around the world to screen retail meat for *C. difficile* (Lund & Peck, 2015; Rodriguez-Palacios et al., 2013a; Zidaric et al., 2010). In a study conducted in Slovenia, *C. difficile* was detected from the fecal samples of 62% of chickens tested. An Austrian study revealed a lower prevalence of 3% *C. difficile* prevalence in 100 ground meat samples collected at retail (Jobstl et al., 2010). The first study investigating the prevalence of *C. difficile* in retail meat in the US was conducted in Tucson, Arizona, which revealed a prevalence of 50% in ground beef, 15% in summer sausage, 43% in ground pork, 44% in ground turkey, and 63% in braunschweiger (Songer et al., 2009). It was found that 73% of the *C. difficile* isolates belonged to ribotype 087, whereas the remaining isolates were of ribotype 027. Although a limited number of studies in the U.S. investigated the prevalence of *C. difficile* in meats, an in-depth characterization of the food isolates has not been performed. The prevalence of *C. difficile* in foods observed in previous investigations varies from 0 to 40% (Harvey et al., 2011a; Limbago et al., 2012; Weese et al.,

2009). However, no studies have been reported from the New England region, including Connecticut, where the highest rate of CDAD was reported in 2010 and 2011 (Steiner et al., 2012).

Besides meats, *C. difficile* was also reported to be present in soil, water, raw vegetable samples, and milk (Hensgens et al., 2012; Janezic et al., 2012a; Jobstl et al., 2010; Kotila et al., 2013; Metcalf et al., 2010; Rodriguez-Palacios et al., 2013a). All these data taken together provide further support to the likelihood of foods, especially those of animal origin, serving as a source of *C. difficile* infection in humans.

In summary, *C. difficile* is a major cause of antibiotic-associated diarrhea in hospital settings. *C. difficile* predominantly affects hospital inpatients undergoing prolonged antibiotic therapy, which results in enteric dysbiosis, leading to *C. difficile* spore germination, pathogen colonization in the intestine and subsequent toxin production. Therapeutic agents that inhibit critical *C. difficile* virulence factors such as toxin production, sporulation, and spore outgrowth without causing enteric dysbiosis could improve the clinical outcome of CDAD and prevent relapse of the infection. Most of the antimicrobials, including anti- *C. difficile* antibiotics are found to predispose patients to CDAD and its relapse by inducing gut dysbiosis. Moreover, the emergence of antibiotic-resistant strains of hypervirulent *C. difficile* is reported worldwide, warranting alternative therapeutic strategies. In addition, the detection of genotypically similar and identical *C. difficile* strains implicated from human infections in foods and food animals suggests the potential role of food as a source of community-associated *C. difficile* disease, which necessitates detailed studies on the prevalence of *C. difficile* in foods and characterization of the food isolates.

Based on published literature and preliminary research conducted in our laboratory, it is hypothesized that meat is a potential source of multi-drug resistant *C. difficile*. Moreover, it was

hypothesized that CR and TC reduce *C. difficile* virulence by reducing toxin production and sporulation without deleteriously affecting the normal enteric flora. The specific objectives of this dissertation include

1. To determine the prevalence of *C. difficile* in retail meat sold in Connecticut, and to characterize *C. difficile* meat isolates by phenotypic and genotypic tests.
2. To investigate the effect of CR and TC on *C. difficile* toxin production *in vitro*.
3. To investigate the effect of CR on *C. difficile* sporulation and spore germination *in vitro*.
4. To study the effect of CR on *C. difficile* pathogenicity in a mouse model.

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Chapter III

The prevalence of *C. difficile* in retail meat sold in Connecticut, and characterization of a *C. difficile* meat isolate by phenotypic and genotypic tests.

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Abstract

Clostridium difficile is a pathogen of significant public health concern causing a life-threatening, toxin-mediated enteric disease in humans. The incidence and severity of the disease associated with *C. difficile* have increased in the US with the emergence of hypervirulent strains and community associated outbreaks. The detection of genotypically similar and identical *C. difficile* strains implicated from human infections in foods and food animals indicate the potential role of food as a source of community associated *C. difficile* disease. One hundred samples each of ground beef, pork and chicken purchased from geographically distant grocery stores in Connecticut were tested for *C. difficile*. Positive isolates were characterized by ribotyping, antibiotic susceptibility, toxin production and whole genome sequencing. Of the 300 meat samples, only two pork samples tested positive for *C. difficile* indicating a very low prevalence of *C. difficile* in meat. The isolates were non-toxigenic; however, genome characterization revealed the presence of several antibiotic resistance genes and mobile elements that can potentially contribute to generation of multidrug resistant toxigenic *C. difficile* by horizontal gene transfer. Further studies are warranted to investigate potential food-borne transmission of the meat isolates and development of multi-drug resistance in these strains.

1. Introduction

Clostridium difficile is a major cause of enteric disease in humans, with fecal-oral route as the primary mode of transmission. *C. difficile* disease has been traditionally regarded as a nosocomial infection in humans, especially in those receiving prolonged antimicrobial therapy (Hookman & Barkin, 2009; McDonald et al., 2006). However, in recent years, an increase in the number and severity of *C. difficile* infection in humans has been reported, particularly in those involving communities outside the hospital environment and low risk subjects (McDonald et al., 2006; Rupnik et al., 2009). Recent studies conducted by investigators worldwide have indicated the occurrence of *C. difficile* in a variety of food animals. A rise in isolation rates of the pathogen from animal reservoirs is one potential reason attributed to the increased reports of human *C. difficile* infections (Rupnik et al., 2009; Thitaram et al., 2011b). In addition, *C. difficile* has been isolated from raw and ready-to-eat meats at retail stores (Harvey et al., 2011b; Songer et al., 2009; Weese et al., 2009; Weese et al., 2010b). The detection of genotypically similar and identical *C. difficile* strains implicated from human infections in foods and food animals (Rodriguez-Palacios et al., 2011; Weese et al., 2011) further strengthens the potential role of food as a source of community associated *C. difficile* disease (Marsh et al., 2011; Songer et al., 2009). Although a limited number of studies in the U.S investigated the prevalence of *C. difficile* in meats, an in-depth characterization of the food isolates has not been performed. The prevalence of *C. difficile* in foods observed in previous investigations varies from 0 to 40% (Harvey et al., 2011a; Limbago et al., 2012; Songer et al., 2009; Weese et al., 2009). A report by the US Department of Health and Human Services reported the highest rate of *C. difficile* associated hospitalizations in the New England region in 2010 and 2011 (Steiner et al., 2012). The Centers for Disease Control and Prevention (CDC) in collaboration with the Connecticut Department of Public Health (DPH) has

initiated the Emerging Infections Program (EIP) for *C. difficile* surveillance with the objectives to (1) determine the population-based incidence of community- and healthcare-associated *C. difficile* infection, and (2) to characterize *C. difficile* strains that are responsible for infection in the population under surveillance, with a focus on strains from community-associated cases.

Recent studies have suggested the possible emergence of new virulent or antibiotic resistant *C. difficile* strains by horizontal gene transfer between toxigenic and non-toxigenic strains (Brouwer et al., 2013). In addition, a closely related foodborne pathogen, *C. perfringens* was found to acquire virulence by way of horizontal gene transfer in the gut environment (Kobayashi et al., 2009; Popoff & Bouvet, 2013). Moreover, the CDC has listed *C. difficile* as one among the three urgent threats in their recent report on emerging pathogens with antibiotic resistance (Steiner et al., 2012). A genome-wide characterization of *C. difficile* food isolates would provide their virulence and antimicrobial resistance profiles, besides indicating the genetic relatedness and clonality between food and clinical isolates.

2. Materials and Methods

2.1 Sample collection and detection of *C. difficile* in meat

A total of 100 samples each of ground beef, ground pork and chicken wings were procured from 23 geographically distant retail stores in Connecticut. All the procured samples were brought to the laboratory on ice and processed within 24 h of receipt. Qualitative detection of *C. difficile* was performed as described previously (Weese et al., 2010b), with modification for a higher detection limit. Fifty grams each of ground beef, ground pork or a chicken wing were separately added to 50 ml of *C. difficile* moxalactam-norfloxacin (CDMN, Oxoid, Hampshire, UK) broth supplemented with 0.1% sodium taurocholate (Sigma-Aldrich, Inc., St. Louis, MO, USA), and subjected to stomaching for 1 minute. After stomaching, 1 ml of the broth was pour-plated in

duplicate to *C. difficile* moxalactam-norfloxacin (CDMN) agar, while the remaining broth-sample mixture was incubated anaerobically at 37°C for 48 h. After incubation, an aliquot of the broth was subjected to alcohol shock by adding 100% anhydrous ethanol for 1 h to eliminate the vegetative bacteria. The broth was subsequently subjected to centrifugation at 4000 x g for 10 min, and the pellet was resuspended in 0.5 ml of phosphate buffered saline (PBS) and pour plated in CDMN agar. The agar plates were incubated anaerobically at 37°C for 48 h. presumptive *C. difficile* colonies were subcultured onto 7% horse blood agar and follow up identification of *C. difficile* was done based on growth in CDMN agar, Gram's staining, and colony characteristics. The identity of *C. difficile* was further confirmed by L-proline aminopeptidase reaction (Pro Disc, Remel, Lenexa, KS, USA) and PCR targeting *tpi*, a species-specific housekeeping gene of *C. difficile* (Lemee et al., 2004).

2.2 Detection of toxins

The isolated *C. difficile* strains were screened for the presence of TcdA/B using a commercially available enzyme immunoassay (EIA) *C. difficile* Tox A/B II kit (TechLab, Blacksburg, VA, USA). The assay was previously optimized in our laboratory to quantitatively detect toxin A/B using a standard curve method (Mooyottu et al., 2014e). The isolates were revived on blood agar and an individual colony was transferred to 10 ml of Brain Heart Infusion broth (BHI). The cultures were incubated 24 h anaerobically at 37°C and pelleted by centrifugation. The supernatant was used for the EIA as per the manufacturer's instructions. *C. difficile* ATCC BAA 1870 was used as a positive control.

2.3 Antibiotic susceptibility tests

The isolated *C. difficile* strains were tested for the susceptibility to 10 antimicrobials (ciprofloxacin, moxifloxacin, clindamycin, erythromycin, ampicillin, ceftiofur, metronidazole,

rifampicin, and vancomycin) using the Etest (bioMérieux, Marcy l'Etoile, France) on Brucella blood agar supplemented with hemin and vitamin K, according to the manufacturer's instructions. The plates were incubated for 48 h at 37°C under anaerobic conditions, after which the minimum inhibitory concentration (MIC) values were determined. The MIC breakpoints for clindamycin, vancomycin, metronidazole and moxifloxacin were compared with those established by the CLSI (Clinical and Laboratory Standards Institute (CLSI), 2007), European Committee for Antimicrobial Susceptibility Testing (EUCAST) and British Society of antimicrobial chemotherapy (BSAC) for *C. difficile*. For assessing the susceptibility for erythromycin, ampicillin, tetracyclin, rifampicin, ciprofloxacin, cefoxitin and clindamycin, the epidemiological cutoff values (ECOFF) recently established by the EUCAST obtained from the EUCAST MIC distribution database (<http://mic.eucast.org/Eucast2/SearchController/search.jsp?action=init>) were used. *C. difficile* ATCC 700057 was used as a control. The *C. difficile* isolates were considered multidrug resistant (MDR) if they were resistant to three or more classes of antimicrobial agents (Schwarz et al., 2010).

In addition, the antibiotic resistance profiles of the *C. difficile* isolates were compared with one clinical isolate (ATCC BAA 1805) and non-toxigenic *C. difficile* strain ATCC 700057 using antibiotic susceptibility discs (Oxoid, USA) in Brucella agar with 5% laked sheep blood supplemented with hemin and vitamin K, as per CLSI directions. The antibiotics screened include ampicillin, tetracycline, erythromycin, rifampicin, clindamycin, vancomycin, erythromycin, cefoxitin, moxifloxacin and ciprofloxacin.

2.4 Toxin gene detection and ribotyping

A Chelex Resin-based DNA extraction kit (InstaGene Matrix, Bio-Rad, France) was used for extracting the genomic DNA of *C. difficile* isolates. A multiplex PCR was carried out to detect

the genes encoding toxins, TcdA, TcdB, CdtA, CdtB and TcdC deletion using previously published primers (Antikainen et al., 2009). The PCR amplification was done using HotstarTaq (Qiagen, Germany) PCR master mix, with thermocycler conditions: 10 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, and a final extension of 5 min at 72°C.

The PCR ribotyping was performed using a previously published protocol (Bidet et al., 1999). The amplification reactions with designed primers were performed using HotstarTaq PCR master mix (Qiagen, USA). The thermocycler conditions were 10 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C, and a final extension of 5 min at 72°C. The PCR products were electrophoresed for 10 h at 75 V in 0.5X TBE using 3% agarose gel (MidSci, St.Louis, MO, USA) stained with 0.5µg/mL ethidium bromide, and analyzed under UV light using the BioDocIT Imaging System (UVP, LLC , Upland , CA , USA). The ribotype profiles were analyzed with GelCompar II image analysis software (version 5.1; Applied Maths., Austin, TX, USA).

2.5 Whole genome sequencing

The whole genome sequencing of the *C. difficile* isolates was done using the Illumina next-generation sequencing (NGS) technology (Illumina, San Diego, CA, USA). The genomic DNA from *C. difficile* cultured on blood agar was extracted using the DNAeasy mini kit (Qiagen, Germany), and multiplexed paired-end libraries (DNA fragments with each sample's DNA tagged with a unique index) were generated. The DNA libraries were sequenced using sequencing-by-synthesis technology, 13 on the Illumina MiSeq platform, generating 300 base paired-end reads. Contigs were assembled using CLC genomics workbench (CLC bio, Qiagen, Germany) and annotated using Rapid Annotation Using Subsystem Technology (RAST, Rast. Nmpdr.org) and NCBI's Prokaryotic Genomes Annotation Pipeline (PGAP). The draft sequence was submitted to

the NCBI- National Center for Biotechnology Information (NCBI) whole genome shotgun (WGS) database.

3. Results and discussion

In this study, we investigated the prevalence of *C. difficile* in 100 samples each of ground beef, ground pork and chicken wings purchased from different geographic locations in Connecticut. We followed direct plating and selective enrichment followed by alcohol shock in CDMN agar. In order to increase the detection limit, a larger volume (1 ml) was pour plated instead of plating 100 µl on to CDMN agar. Also, the whole meat-CDMN broth mixture was enriched for further plating followed by an alcohol shock. Upon direct plating and enrichment, 40% of the samples showed colonies on CDMN agar plates. Gram's staining of these colonies revealed typical *C. difficile* morphology. In addition, proline amino peptidase reaction showed positive reaction in around 20% of the samples. However most of the isolates were confirmed negative for *C. difficile* by PCR for *tpi*. Clostridial species other than *C. difficile*, positive for proline-amino peptidases activity (suggested to be *C. acetobutyrolyticum* or *C. sporogenes* by API 32 tests) were identified using API rapid ID 32 test. No *C. difficile* was isolated from any samples of ground beef and chicken wings. Two of the pork samples tested positive for the presence of *C. difficile*.

Our EIA results indicated the absence of toxins A and B in the isolated cultures. Multiplex PCR for toxins confirmed that the pork isolates were non-toxigenic and negative for toxin A, B and binary toxins. Evidently, no *tcdC* deletions were detected in the two pork isolates. TcdC is a negative regulator in Pathogenicity Locus (PaLoc) of *C. difficile*. Hence TcdC deletion is classically associated with increased toxin production in *C. difficile* strains (Dupuy et al., 2008;

McDonald et al., 2005), and is a characteristic of hyper-virulent human toxigenic strains. However, since the Connecticut *C. difficile* isolate (SG-12) described in this manuscript does not possess PaLoc region in its genome, no bands could be observed in the PCR conducted for detection of TcdC deletion. Both of the isolates were from the same brand and were from the same store. Non-toxigenic *C. difficile* has been isolated from human clinical and nonclinical sources, besides environment as well as animal sources and food (Janezic et al., 2012b; Natarajan et al., 2013). In healthy humans, the prevalence of *C. difficile* carriage has been reported as between 4 and 7.6%, of which non-toxigenic strains accounted for 42–50% of the total isolates (Natarajan et al., 2013). The sources and genetic relationship between human non-toxigenic *C. difficile* and that of animal or environmental origin are poorly studied.

Ribotyping of the two pork isolates revealed that both samples are of the same ribotype, which is unclassified and different from ribotypes 078, 027, 01, PA-01 and PA- 22 which were previously reported in meat samples. Previous studies have shown that clinically relevant *C. difficile* isolates have been isolated from pigs and piglets. *C. difficile* ribotype 078, which has been reported to cause disease in humans, was isolated from pigs and pork products (Debast et al., 2009; Keessen et al., 2011b; Peláez et al., 2013). Also human toxigenic strains were reported from ground pork and other products (Debast et al., 2009; Weese et al., 2011).

Antibiotic susceptibility tests revealed that the *C. difficile* isolated from pork samples were resistant to an array of antibiotics (Table 1). Antibiotic profile of both pork isolates were identical and thus we presented the data as a single isolate. The pork isolates were found to be resistant to vancomycin (MIC > 2 µg/ml) according CLSI and EUCAST guidelines. The isolates were found to be resistant to ciprofloxacin ((MIC > 32 µg/ml) and moxifloxacin (MIC > 6 µg/ml). The later was assumed as per the EUCAST epidemiological cut off (ECOFF) values. Similarly, the isolates

were not susceptible to cefoxitin even at a high concentration (MIC > 256 µg/ml). The MIC of erythromycin (MIC > 3 µg/ml) indicated that the isolates were resistant to erythromycin according to EUCAST ECOFF values. The isolates were highly resistant to tetracycline (MIC > 256 µg/mL) whose ECOFF value was estimated as 0.25 µg/ml according to EUCAST. The MIC of ampicillin was found to be > 1.5 µg/ml which was similar to the previously published MIC of some clinical isolates, and considered to be susceptible as per the CLSI MIC breakpoints for gram positive anaerobes other than *C. difficile*. The disc diffusion test data revealed that the pork isolates are more resistant (based on zone of inhibition) to vancomycin and ampicillin compared to the clinical isolate (ATCC BAA 1805) tested. Interestingly, the isolates were found to be susceptible to metronidazole (MIC > 0.19 µg/mL) and clindamycin (MIC > 1.5 µg/mL). Since the pork isolates exhibited resistance to more than three groups of antibiotics, this isolate was considered as multidrug resistant. Resistance to multiple classes of antibiotics has been reported in meat and animal *C. difficile* isolates in the USA (Harvey et al., 2011b; Rodriguez-Palacios et al., 2013b), with isolation of multidrug resistant *C. difficile* from food animals and food products (Peláez et al., 2013; Pirs et al., 2013).

Whole genome sequencing of one of the pork isolates results showed that the closest fully sequenced relative was *C. difficile* 630, which is a multidrug resistant isolate of clinical origin. Both isolates were found to be 100% identical in sequence and considered as a single isolate further in this study. Interestingly, the phylogenetic tree (Complete phylogenetic tree is given as Figure. 1) constructed using all completed and draft genomes using the NCBI genome blast tool indicated its close similarity to a swine toxigenic and presumably zoonotic strain *C. difficile* 824 and human acute toxigenic strain *Clostridium difficile* CD44 (Figure. 1). Most of the closely related strains were human hyper virulent clinical isolates. In addition, previously characterized non-toxigenic

isolates such as CD37 (Brouwer et al., 2012) were genetically more distant from the Connecticut pork isolate when compared to toxigenic isolates in its clade (Figure. 1).

The pork isolates did not possess the pathogenicity locus of *C. difficile* that contains toxin and toxin regulatory genes, which is consistent with EIA and multiplex PCR data. However, it possessed an array of antibiotic resistance genes including five vancomycin resistance genes, two fluroquinolone resistance genes that included *gyrA* and *gyrB*, four tetracyclin resistance genes, two beta lactamase genes, two macrolide resistance genes and multiple multidrug resistance efflux pump genes. The genome data were found to be consistent with the antibiotic resistance profile of the isolate. Since the pork isolates were found to be closely related to the fully sequenced multi-drug resistant *C. difficile* 630, we compared antibiotic resistance genes of both strains (Table. 2) using RAST. The Connecticut pork isolate was found to possess a higher number of antibiotic resistant genes compared to *C. difficile* 630. Interestingly, *Tn916*, a conjugative transposable element related to dissemination of antibiotic resistance in Gram positive bacteria (Spigaglia et al., 2005) was also present in the Connecticut pork isolate.

The *Tn916* transposon and related elements are widespread in many clinical strains of *C. difficile*, and their ability to mobilize plasmids or other conjugative transposons could be relevant for acquiring multiple antibiotic resistance and other virulence factors by *C. difficile* (Dong et al., 2014; Spigaglia et al., 2005).

In addition to antibiotic resistance genes, the pork isolates also possessed additional resistance to metals such as zinc and mercury, unlike *C. difficile* 630 strain. Two unique multidrug resistance genes (Multidrug and toxin extrusion (MATE) family efflux pump and Multidrug-efflux transporter, major facilitator superfamily (MFS)) were identified in the pork isolate compared to *C. difficile* 630 strain.

Although the prevalence of *C. difficile* detected in meat samples (2 out of 300 samples tested) was very low, and the strains isolated were non-toxigenic; the presence of multidrug resistance genes and transposable elements raise a significant concern. Since *C. difficile* genome is highly mobile (Sebaihia et al., 2006), horizontal gene transfer and large-scale recombination of core genes has shaped the *C. difficile* genome over both short and long time scales (Dingle et al., 2013). Recent studies indicate that non-toxigenic strains of *C. difficile* can become toxigenic by horizontal gene transfer (Brouwer et al., 2013). The major virulence determinants are Clostridial toxins, toxin A (*tcdA*) and toxin B (*tcdB*), encoded within the pathogenicity locus (PaLoc). PaLoc is variably present, even among closely related strains, and thus resembles a mobile genetic element (Dingle et al., 2013). This raises the concern over the potential generation of toxigenic variants of multidrug resistant nontoxigenic strains of *C. difficile* in the gut. The PaLoc acquisition and loss are common in non-toxigenic and toxigenic strains, as indicated by existence of closely related toxigenic and non-toxigenic strains. Phylogenetic information of the pork isolate indicated its close relatedness to the toxigenic isolates in its clade. Conversely, the presence of conjugative transposons like *Tn* 916 in the pork isolates pose a concern because they confer antibiotic resistance and virulence factors to clinical strains of *C. difficile* in the gut. The presence of multidrug resistant *C. difficile* in foods can potentially facilitate intermixing of toxigenic and non-toxigenic strains in the gut which could lead to the emergence of new multidrug resistant *C. difficile* strains.

The low occurrence of *C. difficile* in meat is consistent with a previous studies (Limbago et al., 2012). However, the source of contamination of *C. difficile* in retail meat is not known, which could be from meat processing facilities, facility personal or the animals (Harvey et al., 2011a; Jöbstl et al., 2010; Rodriguez-Palacios et al., 2013b).

4. Conclusion

In summary, we confirmed that *C. difficile* occurs at very low levels in raw meat sold in Connecticut. The isolates were non-toxigenic, but found to have an array of antibiotic resistance genes and mobile elements, which can potentially contribute to the generation of multi-drug resistant toxigenic strains. Further studies are warranted to delineate the source of contamination and the effect of cooking and other processing operations on the viability of *C. difficile* spores in meat.

Nucleotide sequence accession numbers

This Whole Genome Shotgun project has been deposited in DDBJ/EMBL/GenBank under accession number JFAF000000000. The version described in this paper is the first version, JFAF010000000. <http://www.ncbi.nlm.nih.gov/nuccore/JFAF000000000>

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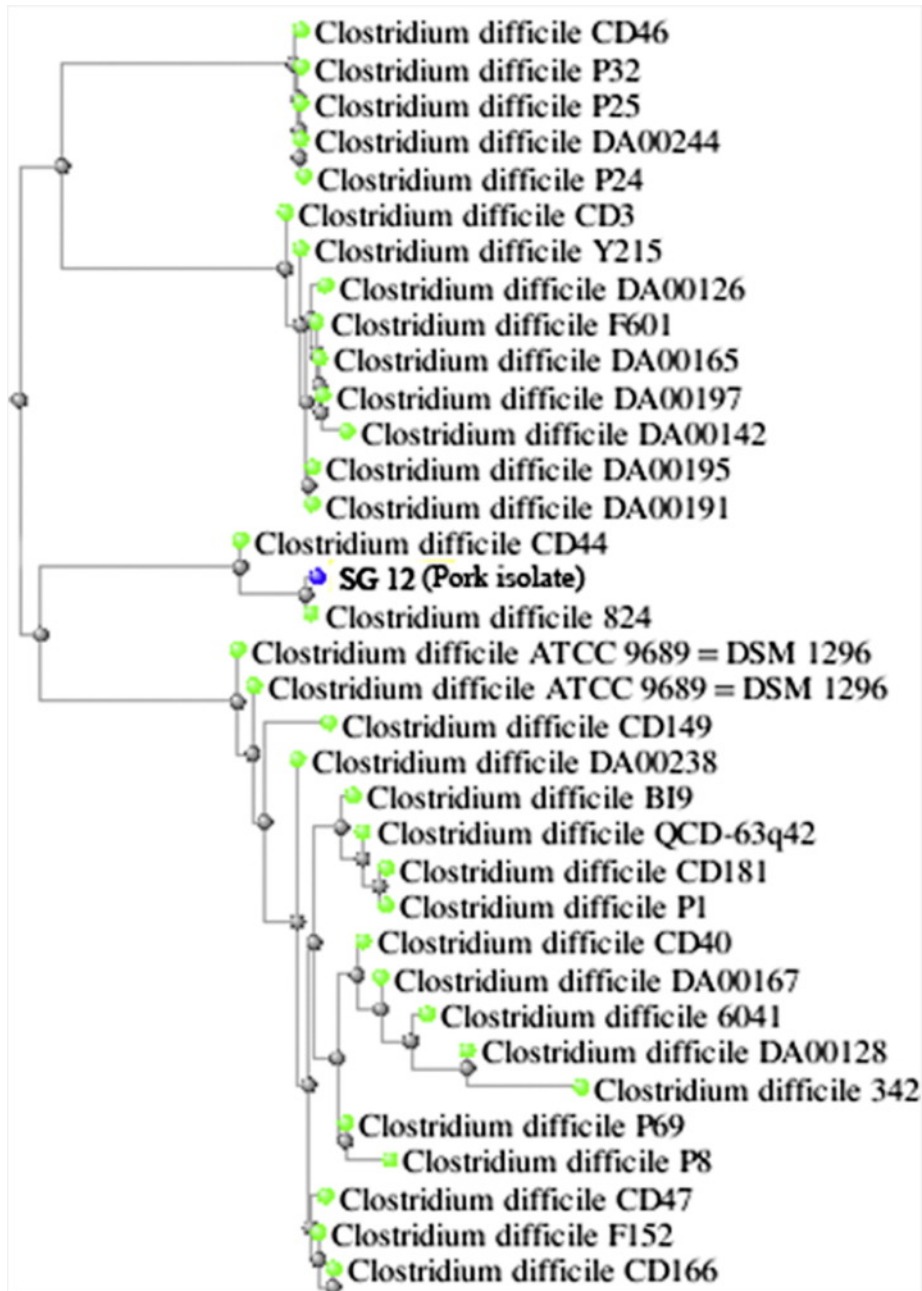
Table 1. Minimum inhibitory concentrations (MIC) values of 10 antimicrobials against the pork isolate of *C. difficile* (SG-12) determined by Etest, and respective Clinical & Laboratory Standards Institute (CLSI) break points and ecological cut-off values (ECOFF) established by The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Zone of inhibition for same antimicrobials for SG-12, clinical isolate ATCC 1870 and non-toxigenic isolate ATCC 700057 estimated using disc diffusion method are also determined.

MIC values and comparative antibiotic resistance profile of <i>C. difficile</i> pork isolate (SG-12).						
Antibiotic	MIC (SG12) µg/ml(E-test)	CLSI break point µg/ml	EUCAST ECOFF(µg/ml) <	Zone of inhibition (SG-12) mm	Zone of inhibition (ATCC BAA 1870) mm	Zone of inhibition (ATCC 700057) mm
Ciprofloxacin	> 32	–	–	0	0	0
Moxifloxacin	6	–	4	2	2	0
Vancomycin	> 2	> 2	2	23	35	16
Tetracyclin	> 256	–	0.25	7	38	10
Erythromycin	3	–	2	23	0	38
Cefoxitin	> 256	–	–	0	0	0
Clindamicin	> 1.5	–	16	1.2	7	0
Metronidazol	> 1.9	2	2	25	2	20
Rifampicin	< 0.02	–	0.004	30	25	26
Ampicillin	1.5	–	–	23	25	27

Table 2. Antibiotic resistance genes identified in the genome of pork isolate SG-12 and multidrug resistant *C. difficile* 630 (CD-630). The presence or absence of antibiotic resistance genes in annotated *C. difficile* pork isolate (SG-12) were compared with multidrug resistant *C. difficile* 630 (CD-630) reference genome using RAST.

Antibiotic resistance genes identified in <i>C. difficile</i> pork isolate (SG-12) and reference strain <i>C. difficile</i> 630 (CD-630).			
Antibiotic	Resistance genes	SG-12	CD-630
Vancomycin	Vancomycin B-type resistance protein VanW	+	+
Vancomycin Teicoplanin A-type resistance protein VanA	+	+	
Vancomycin response regulator VanR	+	+	
Sensor histidine kinase VanS	+	+	
<i>Streptococcus pneumoniae</i> Vancomycin Tolerance Locus Vex 2	+	–	
<i>Streptococcus pneumoniae</i> Vancomycin Tolerance Locus Vex 3	+	–	
Two-component response regulator VncR	+	–	
Sensor histidine kinase VncS	+	–	
Fluoroquinolones	DNA gyrase subunit A	+	+
DNA gyrase subunit B	+	+	
Tetracycline	Tetracycline resistance protein TetM	+	+
Translation elongation factor G	+	+	
Beta-lactam antibiotics	Beta-lactamase repressor BlaI	+	–
Regulatory protein BlaR-1	+	+	
Beta-lactamase BL	+	–	
Macrolides	Macrolide efflux protein	+	+
Macrolide glycosyltransferase	+	+	
Multidrug resistance	Multi antimicrobial extrusion protein (Na ⁺)/drug antiporter<comma> MATE family of MDR efflux pumps	+	+
Multidrug and toxin extrusion (MATE) family efflux pump YdhE/NorM<comma> homolog	+	–	
Multidrug-efflux transporter<comma> major facilitator superfamily (MFS)	+	–	

Figure 1. Phylogenetic tree constructed by genome blast (NCBI) demonstrating relationship between the pork isolate SG-12 and other complete and draft genomes. Phylogenetic tree was generated by minimum evolution method.



Chapter IV

Effect of carvacrol (CR) and *trans*-cinnamaldehyde (TC) on *C. difficile* toxin production, cytotoxicity and toxin gene expression

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Abstract

Clostridium difficile is a nosocomial pathogen that causes a serious toxin-mediated enteric disease in humans. Reducing *C. difficile* toxin production could significantly minimize its pathogenicity and improve disease outcomes in humans. This study investigated the efficacy of two, food-grade, plant-derived compounds, namely *trans*-cinnamaldehyde (TC) and carvacrol (CR) in reducing *C. difficile* toxin production and cytotoxicity *in vitro*. Three hypervirulent *C. difficile* isolates were grown with or without the sub-inhibitory concentrations of TC or CR, and the culture supernatant and the bacterial pellet were collected for total toxin quantitation, Vero cell cytotoxicity assay and RT-qPCR analysis of toxin-encoding genes. The effect of CR and TC on a *codY* mutant and wild type *C. difficile* was also investigated. Carvacrol and TC substantially reduced *C. difficile* toxin production and cytotoxicity on Vero cells. The plant compounds also significantly down-regulated toxin production genes. Carvacrol and TC did not inhibit toxin production in the *codY* mutant of *C. difficile*, suggesting a potential *codY*-mediated anti-toxigenic mechanism of the plant compounds. The antitoxigenic concentrations of CR and TC did not inhibit the growth of beneficial gut bacteria. Our results suggest that CR and TC could potentially be used to control *C. difficile*, and warrant future studies *in vivo*.

1. Introduction

Clostridium difficile is a gram-positive, spore-forming, anaerobic bacterium that causes a toxin-mediated enteric disease in humans (Weese, 2010). More than 300,000 cases of *C. difficile*-associated disease (CDAD) are reported annually in the United States, resulting in approximately US\$3 billion of health care costs (Ghose et al., 2007; Wilkins & Lyerly, 2003). The emergence of a hypervirulent strain, NAP1/ribotype 027, that produces increased levels of toxins and a severe form of the disease in humans has been reported (Blossom & McDonald, 2007; Hookman & Barkin, 2009; Sunenshine & McDonald, 2006). *C. difficile* predominantly affects long-term hospital inpatients and the elderly undergoing prolonged antibiotic therapy (Hookman & Barkin, 2009). Prolonged antibiotic therapy results in the disruption of the normal enteric microflora, leading to the germination of *C. difficile* spores and pathogen colonization in the intestine with subsequent production of toxins (Dial et al., 2005). The toxins, TcdA and TcdB, act as glucosyl transferases that inactivate the Rho family GTPases associated with F-actin regulation, and consequently cause disruption of the cytoskeleton and intestinal epithelial tight junctions (Keel & Songer, 2006; von Eichel-Streiber et al., 1999). This leads to an inflammatory response with the release of cytokines and leukotrienes, causing pseudomembrane formation and watery diarrhea (Hookman & Barkin, 2009; McDonald et al., 2006; Sunenshine & McDonald, 2006). The genes *tcdA* and *tcdB*, which encode the *C. difficile* toxins TcdA and TcdB, respectively, along with the genes encoding TcdR, an RNA polymerase sigma factor needed for maximal expression of *tcdA* and *tcdB*, TcdC, an antagonist of TcdR, and TcdE, a holin-like protein needed for toxin excretion, constitute the “pathogenicity locus” in *C. difficile* (McDonald et al., 2006). Expression of the pathogenicity locus is controlled by a number of environmental factors, including the availability of rapidly metabolizable carbon sources, a regulation mediated by the global regulator CcpA

(Antunes et al., 2011a; Antunes et al., 2012) and the intracellular pools of the branched-chain amino acids and GTP, mediated by the global regulator CodY (Dineen et al., 2007; Dineen et al., 2010). Although exposure to broad-spectrum antibiotics predisposes patients to CDAD by disrupting the normal gut flora (Bartlett, 1992; O'Connor et al., 2004), antibiotics are still the primary line of treatment for patients who have contracted the disease. In addition, the emergence of antibiotic resistance in hypervirulent strains of *C. difficile* is increasingly reported worldwide (Prabaker & Weinstein, 2011; Spigaglia et al., 2011). Since *C. difficile* toxins are the major virulence factors responsible for the pathogenesis of CDAD, identification of alternative therapeutic agents that inhibit *C. difficile* toxin production without affecting the normal gastrointestinal flora or exacerbating bacterial antibiotic resistance could be a potentially viable approach for controlling CDAD.

Historically, plants have been used for treating various diseases in traditional medicine (Johny et al., 2010). Carvacrol (CR) is a monoterpenoid phenol present in oregano and thyme oils. Diverse pharmacological actions of CR, including antimicrobial and anti-inflammatory properties, have been previously demonstrated (Baser, 2008). *Trans*-cinnamaldehyde (TC) is an aromatic aldehyde present as a major component of the bark extract of cinnamon. Previous research conducted in our laboratory revealed that sub-inhibitory concentrations (SICs; the concentrations that do not inhibit bacterial growth) of TC and CR increased the sensitivity of multi-drug resistant *Salmonella* Typhimurium DT 104 to antibiotics by down-regulating antibiotic resistance genes and the efflux pump, *tolC* (Johny et al., 2010). In addition, we previously observed that TC inhibited biofilm synthesis and virulence in uropathogenic *Escherichia coli* (Amalaradjou et al., 2010; Amalaradjou et al., 2011). The objective of this study was to investigate the effect of sub-inhibitory

concentrations (SICs) of TC and CR on toxin production and cytotoxicity of *C. difficile in vitro*, and delineate the potential mechanism(s) behind their effect.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Four hypervirulent *C. difficile* isolates (ATCC# BAA 1870, 1053, 1805 and UK1) were grown in brain heart infusion broth (BHI) supplemented with 5% yeast extract (Difco, Sparks, MD, USA) in a Whitley A35 anaerobic work station (Microbiology Inc., Frederick, MD, USA) in the presence of 80% nitrogen, 10% carbon dioxide and 10% hydrogen at 37 °C for 24 h. The bacterial population was titered by plating 0.1 mL portions of appropriate dilutions on BHI agar and *Clostridium difficile* moxalactum norfloxacin (CDMN) agar (Oxoid, Hampshire, UK) supplemented with 5% horse blood, under strict anaerobic conditions at 37 °C for 24 h. In addition, seven selected beneficial enteric bacteria obtained from the USDA-ARS culture collection, Peoria, IL (*Lactobacillus brevis*, *L. reuterii*, *L. delbrueckii bulgaricus*, *L. fermentum*, *L. plantarum*, *Bifidobacterium bifidum*, and *Lactococcus lactis lactis*) were separately grown in de Man, Rogosa and Sharpe (MRS) broth (Difco, Sparks, MD, USA) under anaerobic conditions at 37 °C. The titers of the cultures were determined by plating 0.1 mL portions of appropriate dilutions on MRS agar (Difco, Sparks, MD, USA) with incubation at 37 °C for 24 h. The cultures were sedimented by centrifugation (3600 g, 15 min, 4 °C), and the pellets were washed twice, and resuspended in sterile phosphate-buffered saline (pH 7.3), and used as the inoculum.

2.2. Plant Compounds and SIC Determination

The SIC of TC and CR was determined as previously described (Johny et al., 2010). Fifty ml of BHI supplemented with 5% yeast extract was inoculated separately with ~5.0 log₁₀ CFU of each

C. difficile isolate, followed by the addition of 1 to 10 µL of TC or CR (Sigma-Aldrich, St. Louis, MO, USA) with an increment of 1 µL. The cultures were incubated at 37 °C for 24 h, and bacterial growth was determined by measuring the optical density at 600 nm. The highest concentration of each plant compound that did not inhibit bacterial growth after 24 h of incubation was selected as its respective SIC (Sub Inhibitory Concentration) for this study.

Similarly, the effect of SIC of TC and CR on the growth of the aforementioned beneficial gut bacteria was determined by culturing them separately in 10 mL of MRS broth under anaerobic conditions at 37 °C with or without the plant compounds for 24 h. The growth of each culture was determined by measuring optical density at 600 nm, and plating on MRS agar.

2.3. Effect of Plant Compounds on *C. difficile* Toxin Production and Cytotoxicity

Brain Heart infusion broth, with or without the SIC of TC or CR was inoculated (10^5 CFU/mL) separately with each *C. difficile* isolate, and incubated at 37 °C for 48 h anaerobically as before. The culture supernatant was collected at 15, 24, and 48 h of incubation for total toxin A and B quantitation by ELISA (Merrigan et al., 2010) and for determining cytotoxicity on Vero cells. The bacterial pellets were harvested at

6 h and 12 h for RNA isolation and RT-qPCR analysis of *C. difficile* genes associated with toxin synthesis.

2.4. ELISA for Total Toxin A and B

The amount of toxin in the culture supernatant was quantified using the Wampole Tox A/B II kit (TechLabs, Inc., Blacksburg, VA, USA), as described by Merrigan et al. (Merrigan et al., 2010). Purified toxin B (Sigma Aldrich, St. Louis, MO, USA) was used to plot a standard curve. The culture supernatants were diluted and ELISA was performed according to the manufacturer's

instructions. The optical density was measured at 450 nm, compared with the linear range of standard curve, and total toxin concentration was estimated.

2.5. Cytotoxicity Assay

The effect of CR and TC on the cytotoxicity of *C. difficile* culture supernatant was estimated by Vero cell cytotoxicity assay, as described previously (Baines et al., 2005). *C. difficile* culture supernatant was serially diluted (1:10) and added onto Vero cell monolayers in 96-well microtiter plates. The plates were incubated at 37 °C in a 5% CO₂ environment for 48 h, and examined under an inverted microscope. Positive reactions were indicated by the characteristic rounding of Vero cells accompanied by parallel neutralization of cytotoxicity with *Clostridium sordellii* antitoxin (TechLabs, Inc., Blacksburg, VA, USA). The cytotoxicity titer was expressed as the reciprocal of the highest dilution that caused more than 80% cell rounding.

2.6. Real Time Quantitative PCR (RT-qPCR)

In order to determine the effect of CR and TC on *C. difficile* genes involved in toxin synthesis, total RNA was isolated from the early stationary phase (12 h) cultures (Blossom & McDonald, 2007). The culture supernatant was harvested by centrifugation at 3000 × g for 10 min at 4 °C. The bacterial pellet was resuspended in RNAwiz solution (Ambion, Austin, TX, USA), flash frozen in liquid nitrogen, and stored at −80 °C. Total RNA extraction was performed using the Ambion RiboPure Bacteria RNA kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions, followed by DNase I digestion using Turbo DNase I (Ambion). The RNA obtained after each DNase I digestion was purified further using the Qiagen RNeasy RNA column purification kit, according to the manufacturer's instructions (Qiagen, Germantown, MD, USA). The cDNA was synthesized using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). RT-qPCR analysis of the genes associated with toxin production was performed using

published primers for Paloc genes (Vohra & Poxton, 2011) normalized against 16S rRNA gene expression. Twenty-five µL reactions were performed in triplicate using iTaq SYBR (Bio-Rad, Hercules, CA, USA). The relative fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.7. Construction of a *codY* Mutant of *C. difficile* Strain UK-1 and the Effect of Plant Compounds on *codY* Mutant and Parental Strains

The pJS107 plasmid (Francis et al., 2013) was used as a TargetTron vector to introduce mutations into the NAP1/027 *C. difficile* strain UK-1. The group II intron insertion sites for *C. difficile* *codY* were identified using an algorithm that can be found at http://dna.med.monash.edu.au/~torsten/intron_site_finder/. The intron fragment was generated by PCR using primers oLB178 (TGAACGCAAGTTTCTA ATTTTCGATTGTAGTTCGATAGAGGAAAGTGTCT), oLB179 (AAAAAAGCTTATAATTAT CCTTA ACTACCGTAGTAGTGCGCC CAGATAGGGTG), oLB180 (CAGATTGTACAAATGTGGTGATAACAGATAAGTCGTAGTATATAACTTACCTTTCTT TGT) and EBS-Universal (CGAAATTAGAACTTGCGTTCAGTAAAC), and a 1:1 mixture of pBL64 and pBL65 as template (Bouillaut et al., 2013) and then cloned at the *Hind*III and *Bsr*GI sites of pJS107, yielding pBL103. pBL103 was then introduced into *B. subtilis* strain BS49 by transformation. Strain BS49 carries a conjugative transposon, Tn916, integrated into its chromosome and serves as a donor in conjugation with *C. difficile*. Conjugation experiments were carried out as described previously (Carter et al., 2011). *C. difficile* transconjugants were selected on thiamphenicol (10 µg/mL) and then screened for the presence of Tn916 by assaying tetracycline resistance (10 µg/mL). Thiamphenicol-resistant, tetracycline-sensitive (plasmid-containing, transposon-negative) transconjugants were selected for further use. Potential TargetTron mutants

were identified by plating on lincomycin (20 µg/mL) and screening for the insertion of the intron into *C. difficile codY* by PCR using primers specific for full-length *C. difficile codY*. (oLB205, TGAGCATGCTTAATGATGATGATGATGATGTTGATTGTTTTTTAATTTTTTTAATTCA TC; and oLB206, TATCCGGAATTCTGAGGAGATGATTAAATGGC), the 5' intron insertion site and the 3' intron insertion site and a positive clone, strain LB-CD16, was identified. A Southern blot was performed as previously described (Bouillaut et al., 2013) to confirm that the mutant contained a single insertion of the intron.

The role of *codY* on the anti-toxigenic effect mediated by the plant compounds was investigated by quantifying toxin production and expression of toxin-associated genes in CR- and TC-treated *codY* mutant and wild type strains of *C. difficile*. Brain Heart infusion broth, with or without the SIC of TC or CR was separately inoculated (10^5 CFU/mL) with UK1 or LB16, and incubated at 37 °C for 48 h, as described previously. The culture supernatants were collected at 24 and 48 h of incubation for total toxin A and B quantitation by ELISA. In addition, the bacterial pellets were harvested at 12 h for RNA isolation and RT-qPCR analysis.

2.8. Statistical Analysis

All experiments had duplicate samples and the study was repeated three times. The data were analysed using the PROC-MIXED procedure of SAS v. 9.3 (SAS Institute Inc, Cary, NC, USA). Differences between the means were considered significantly different at $p < 0.05$.

3. Results

3.1 Sub-Inhibitory Concentrations of CR and TC

The SICs of CR and TC were found to be 0.60 mM (1.0 mg/L) and 0.38 mM (0.5mg/L), respectively. These concentrations of plant compounds did not inhibit the growth of all *C. difficile* isolates including CodY mutant and parental strains after 24 h incubation at 37 °C. In addition, the OD600 values for the seven selected beneficial bacterial isolates cultured in the presence of SICs of CR and TC were not significantly different from their respective controls (bacteria grown without CR or TC, Figure 1). This indicated that the concentrations of CR and TC used in this study were non-inhibitory to the growth of *C. difficile* as well as the seven selected beneficial bacteria.

3.2 Effect of CR and TC on *C. difficile* Toxin Production

The effect of CR and TC on *C. difficile* toxin production was determined by ELISA, as described previously (Merrigan et al., 2010). Carvacrol and TC significantly reduced toxin production in all three *C. difficile* isolates at 24 and 48 h ($p < 0.05$) compared to their controls. At 48 h, CR and TC inhibited the toxin production by approximately 60% and 80%, respectively in isolates BAA 1870 and 1053 (Figure 2A,B). In isolate BAA 1805, CR and TC reduced toxin production by approximately 55% at 48 h, compared to the control (Figure 2C). In isolates BAA 1870 and 1503, TC was more effective in reducing the toxin production than CR ($p < 0.05$) (Figure 2A,B), whereas both compounds exerted a similar inhibitory effect on toxin production in BAA 1805 after 48 h of incubation (Figure 2C).

3.3. Effect of CR and TC on *C. difficile* Toxin-Mediated Cytotoxicity

To determine the efficacy of TC and CR in reducing *C. difficile*-induced cytopathic effects, a cytotoxicity assay was conducted on Vero cells following a previously published protocol (Baines

et al., 2005). Exposure to TC and CR significantly reduced the ability of *C. difficile* culture supernatant to produce cytotoxicity on Vero cells compared to culture supernatants of untreated cells ($p < 0.05$). The cytotoxicity titer in the cells inoculated with CR- and TC-treated *C. difficile* culture supernatant was reduced by ~90% at 48 h in comparison to that in the control (Figure 3).

3.4. Effect of CR and TC on *C. difficile* Toxin Genes

To investigate if the observed reduction in *C. difficile* toxin production was due to modulation of the expression of toxin-associated genes, transcriptional analysis by RT-qPCR (Real time-quantitative polymerase chain reaction) of the genes known to be involved in toxin production and regulation was performed. The data revealed a modulation of *C. difficile* toxin regulatory genes by CR and TC at early stationary phase (12 h) (Figures 4 and 5). Carvacrol significantly down-regulated ($p < 0.05$) the expression of toxin genes *tcdA* and *tcdB* in all three isolates of *C. difficile* ($p < 0.05$) by ~2.5 fold (Figure 4A,C). However, in isolate BAA 1053, the genes were down-regulated by 15 to 25 fold (Figure 4B). Similar results were obtained in *C. difficile* isolates exposed to TC (Figure 5A,B). However, in isolate BAA 1805, no significant difference was observed in comparison to the control (Figure 5C). A significant down-regulation was also observed in the expression of the toxin excretion gene *tcdE* and the positive regulator *tcdR* in two *C. difficile* isolates (ATCC # BAA 1870, 1053) treated with CR and TC (Figures 4 and 5). In isolates BAA 1870 and 1805, *trans*-cinnamaldehyde treatment increased expression of *tcdC*, which encodes the TcdR antagonist (Figure 5A,B). Up-regulation of *tcdC* was observed in CR-treated BAA 1870 and 1053 as well (Figure 4A,B).

3.5. Effect of CR and TC on a *codY* Mutant of *C. difficile*

In this experiment, we treated the *C. difficile codY* mutant (LB-CD16) and its parental strain, UK-1, with the SIC of CR or TC, and determined their effects on toxin production and toxin gene expression. The total toxin quantitation by ELISA (Enzyme Linked Immunosorbent Assay) revealed a significant reduction in toxin production in CR- or TC-treated UK-1 ($p < 0.05$) compared to the control (Figure 6). However, in a *codY* mutant, the level of toxin production in the absence of drug was higher than in the parent strain and neither CR nor TC caused any inhibition of toxin production. In fact, TC treatment enhanced the toxin production in the *codY* mutant strain significantly ($p < 0.05$) compared to the untreated control. Thus, in the absence of functional CodY protein, neither CR nor TC could inhibit toxin production.

RT-qPCR analysis of the toxin genes in the *codY* mutant strain and its parent revealed a trend in gene expression similar to that seen for toxin production (Figure 7). A significant down-regulation of *tcdA* and *tcdB* was observed in strain UK-1 treated with CR or TC ($p < 0.05$). However, consistent with the ELISA results, CR and TC did not cause any down-regulation of the *tcdA* and *tcdB* genes in the *codY* mutant ($p < 0.05$). Specifically, no significant change in the expression of *tcdA*, *tcdB*, *tcdC* and *tcdR* was observed in the *codY* mutant treated with CR. However, TC treatment resulted in a significant up-regulation of *tcdA* and *tcdB* in the mutant ($p < 0.05$). These data collectively suggest that the SICs of CR and TC were unable to exert any inhibitory effect on the toxin-encoding genes in the absence of a functional *codY*.

4. Discussion

C. difficile colonizes the large intestine of patients undergoing prolonged antibiotic therapy, and produces toxins TcdA and TcdB, resulting in CDAD. *C. difficile* toxins lead to inflammation in the intestine, increased epithelial permeability (Castagliuolo et al., 1998; Feltis et al., 2000; He et al.,

2002), enhanced cytokine and chemokine production (Castagliuolo et al., 1998; He et al., 2002), neutrophil infiltration (Kelly et al., 1994) and the release of reactive oxygen intermediates (He et al., 2002), thereby causing direct damage to the intestinal mucosa (Ng et al., 2010). Therefore, reducing toxin production by *C. difficile* is critical in controlling CDAD.

A potential alternate strategy for controlling microbial infections is the use of anti-virulence drugs. This class of drugs aims at reducing bacterial virulence rather than inhibiting bacterial growth (Rasko & Sperandio, 2010) and presents a lesser selective pressure for development of bacterial antimicrobial resistance (Cegelski et al., 2008; Hughes & Sperandio, 2008; Hung et al., 2005). Therefore, in the current study, we investigated the efficacy of SICs of CR and TC as alternative therapeutic agents that can ameliorate CDAD pathology by reducing *C. difficile* toxin production without affecting the growth of the normal intestinal flora. Since the SICs of antimicrobials, including antibiotics, can modulate bacterial gene expression and physico-chemical functions, they have been used for studying effects on bacterial gene expression and virulence (Tsui et al., 2004).

In the current study, we found that the SICs of CR and TC for *C. difficile* growth did not inhibit the growth of seven different species of bacteria commonly found in the human gastrointestinal tract although CR and TC have antimicrobial activity at higher concentrations. The tested gut bacteria included *Lactobacilli* and *Bifidobacteria*, which are the major groups of beneficial gut flora that play a significant role in maintaining normal gut health (Rupa & Mine, 2012). Previous studies have reported that low concentrations of CR and TC exerted no adverse effects on endogenous bacterial populations, including *Lactobacilli* and *Bifidobacteria*, in pigs and poultry (Jamroz et al., 2005; Si et al., 2006).

Our ELISA results indicated that both plant compounds decreased the total toxin production in all three *C. difficile* isolates obtained from the ATCC, compared to their untreated control cultures (Figure 2). In addition, culture supernatants from TC- or CR-treated cells of all three strains had significantly reduced cytotoxic effects on Vero cells (Figure 3), confirming the ELISA results. Previously, Ultee and Smid (2001) reported that sub-MIC concentrations of CR decreased toxin production in *Bacillus cereus* (Ultee & Smid, 2001). Moreover, prior research conducted in our laboratory revealed that plant compounds, including CR and TC, reduce the virulence of *Listeria monocytogenes* by decreasing its attachment to and invasion of cultured human intestinal epithelial and brain micro-vascular endothelial cells by modulating the expression of several virulence factors, including the global regulator, *prfA* (Upadhyay et al., 2012). Since SICs of CR and TC were used in the experiments, the reduction observed in *C. difficile* toxin production in the treated samples was not due to growth inhibition of the bacterium by the plant compounds, but could be due to their potential abilities to modulate the expression of virulence genes associated with toxin production. Therefore, we used RT-qPCR to determine the effect of TC and CR on the transcription of major genes reported to play a role in toxin synthesis and secretion. Our RT-qPCR data revealed a 3-to-20-fold reduction in the expression of toxin-encoding genes *tcdA* and *tcdB* in strains BAA 1870 and BAA 1053. Furthermore, *tcdR*, which encodes a factor essential for high-level toxin production, was down-regulated 3-to-40-fold by CR and TC in those strains (Figures 4 and 5). Down-regulation of TcdR is known to lead to reduced toxin production (Mani et al., 2002). Down-regulation of *tcdE* was also observed in these *C. difficile* isolates treated with CR or TC (Figures 4 and 5). Multiple studies have demonstrated that *tcdE* is critical for toxin release from *C. difficile*, helping to explain the reduced amount of toxins in CR- and TC-treated culture supernatants. In strain BAA 1805, however, the correlation between toxin and holin gene

expression and toxin production was less clear suggesting that in this strain the modulation of toxin synthesis is mediated by a different mechanism than in other strains. Our results show that the compounds block toxin production in all strains, albeit to different extents, and that this effect can be attributed to decreases in toxin gene transcription in all strains except in the case of strain BAA 1805 in presence of TC.

CodY, a global regulatory protein that senses the intracellular levels of branched-chain amino acids and GTP, monitors the nutritional status and regulates metabolism and stress responses in Gram-positive bacteria (Dineen et al., 2010; Guedon et al., 2001; Pohl et al., 2009). In addition, CodY acts as a global regulator of virulence; a *codY* deletion results in enhanced virulence in *Staphylococcus aureus* and other species (Montgomery et al., 2012; Pohl et al., 2009; Rivera et al., 2012; Stenz et al., 2011). In *C. difficile*, CodY is a potent repressor of *tcdR*, thereby preventing expression of the *Paloc* genes when cells are growing rapidly (Dineen et al., 2007; Dineen et al., 2010). Previous studies have shown increased expression of toxin genes in a *codY* mutant of *C. difficile* (Dineen et al., 2007; Dineen et al., 2010). Our results show that CR and TC reduce toxin production and pathogenicity locus gene expression in NAP1/027 strain UK-1, but not in a *codY* null mutant of UK-1. In fact, TC caused hyperexpression of toxin locus genes in the *codY* mutant strain. In a *codY* mutant strain, the compounds lose their effectiveness as inhibitors, suggesting that the compounds work by a mechanism that may affect either CodY synthesis or activity. CodY activity would be reduced if the compounds caused the pools of branched chain amino acids or GTP to drop (Dineen et al., 2007; Dineen et al., 2010). These data collectively suggest that the antitoxigenic effect of CR and TC is mediated at least in part through CodY.

4. Conclusions

To conclude, our study demonstrated that CR and TC were effective in significantly reducing *C. difficile* toxin production. Although there were variations in the expression of toxin encoding genes, both plant molecules reduced toxin production in all strains (BAA 1870, BAA1053, BAA1805 and UK-1). The major toxin encoding genes, *toxA*, and *toxB* were significantly down-regulated in all the tested strains by CR, whereas TC down-regulated these genes in all strains except one (BAA 1805). Moreover, the two plant compounds at SIC concentrations did not inhibit the growth of major gut microflora in humans. The anti-toxigenic effect of CR and TC appears to be mediated through CodY. The results suggest the potential use of TC and CR to attenuate *C. difficile* virulence; *in vivo* studies on the effect of CR and TC on CDAD are thus warranted.

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Figure 1. Effect of sub-inhibitory concentrations of carvacrol (CR) and trans-cinnamaldehyde (TC) on beneficial gut bacteria

Seven selected beneficial gut bacteria (*Lactobacillus brevis* (A), *L. reuterii* (B), *L. delbrueckii bulgaricus* (C), *L. fermentum* (D), *L. plantarum* (E), *Bifidobacterium bifidum* (F), and *Lactococcus lactis lactis* (G)) were grown in de Man, Rogosa and Sharpe broth in anaerobic condition at 37 °C with and without SICs of CR and TC (Control-Ctrl, open square, Carvacrol- CR, closed diamond, *trans*-cinnamaldehyde-TC, open triangle) for 24 h. The bacterial growth was monitored by measuring optical density at 600 nm measured at 6, 12 and 24 h. TC- or CR-treated gut bacterial populations did not change significantly from the controls ($p > 0.05$).

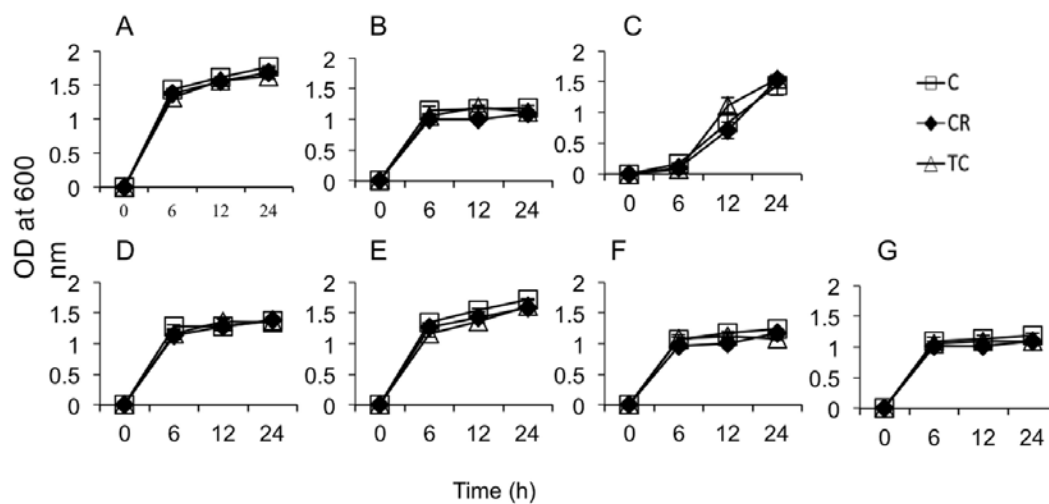
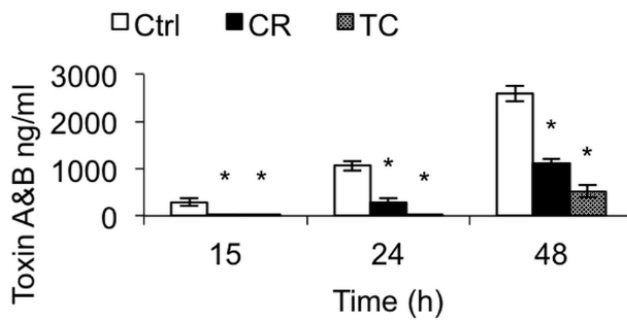


Figure 2. Effect of carvacrol (CR) and *trans*-cinnamaldehyde (TC) on *C. difficile* toxin production. * $p < 0.05$ vs. CR.

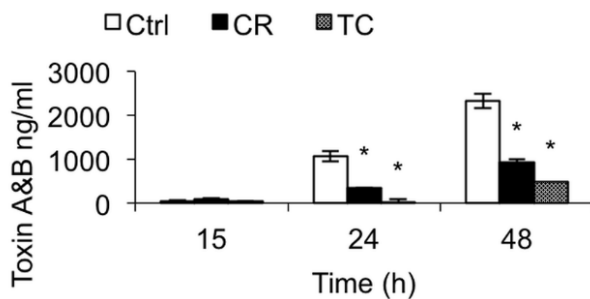
Brain heart infusion with or without the Sub-inhibitory concentration (SIC) of TC or CR, (0.38 and 0.60 mM respectively) was inoculated (10^4 CFU/mL) separately with three hypervirulent *C. difficile* isolates, ATCC BAA 1870 (A), ATCC BAA 1053 (B) or ATCC BAA 1805 (C), and incubated anaerobically at 37 °C for 72 h. The culture supernatant from groups TC, CR and Control (Ctrl) was collected at 15, 24, and 48 h of incubation for total toxin A and B quantitation by ELISA.

* Treatments significantly differed from the control ($p < 0.05$).

A



B



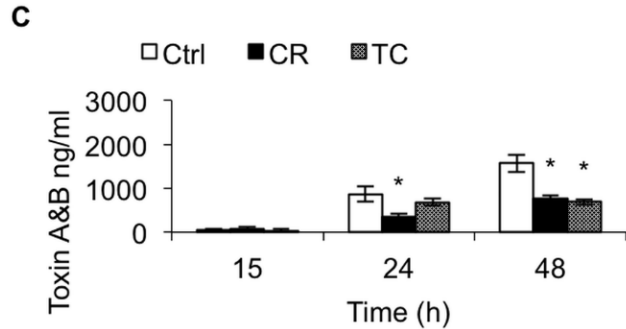
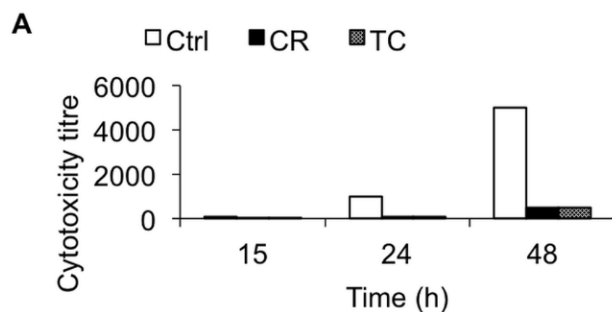


Figure 3. Effect of carvacrol (CR) and *trans*-cinnamaldehyde (TC) on *C. difficile* induced cytotoxicity on Vero cells.

Brain heart infusion with or without the Sub-inhibitory concentration (SIC) of TC or CR, (0.38 and 0.60 mM respectively) was inoculated (105 CFU/mL) separately with three hypervirulent *C. difficile* isolates, ATCC BAA 1870 (A), ATCC BAA 1053 (B) or ATCC BAA 1805 (C), and incubated anaerobically at 37 °C for 72 h. The culture supernatant from groups TC, CR and Control (Ctrl) were collected at 15, 24, and 48 h of and the cytotoxicity titer on Vero cells was determined. Serially diluted *C. difficile* culture supernatants were added to the monolayers in 96-well plates and incubated at 37 °C under 5% CO₂ for 48 h. The cell morphology was examined under an inverted microscope for characteristic rounding as an indication of cytotoxicity.



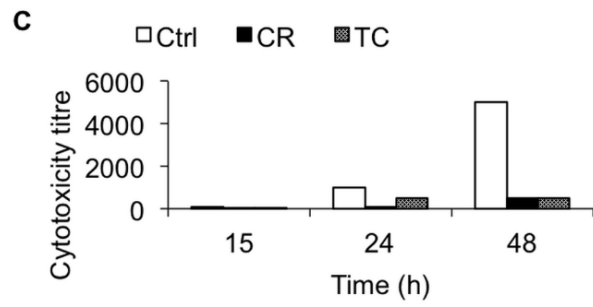
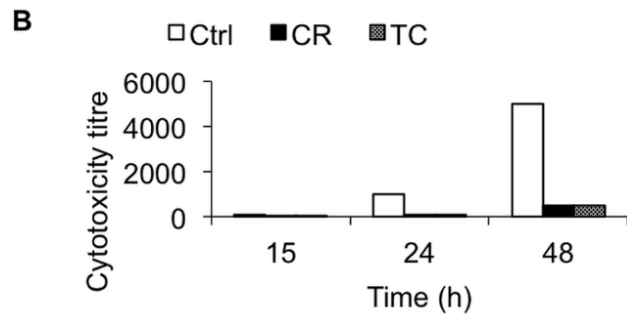


Figure 4. Effect of carvacrol (CR) on *C. difficile* toxin regulatory genes.

Brain heart infusion with or without the sub-inhibitory concentration (SIC) of CR, (0.60 mM) was inoculated (10⁵ CFU/mL) separately with three hyper virulent *C. difficile* isolates, ATCC BAA 1870 (A), ATCC BAA 1053 (B) or ATCC BAA 1805 (C), and incubated anaerobically at 37 °C for 72 h. Bacterial pellets were harvested at 6 h and 12 h for RNA isolation and RT-qPCR for toxin regulatory genes. * Treatments significantly differed from the controls at 24 and 48 h of incubation ($p < 0.05$).

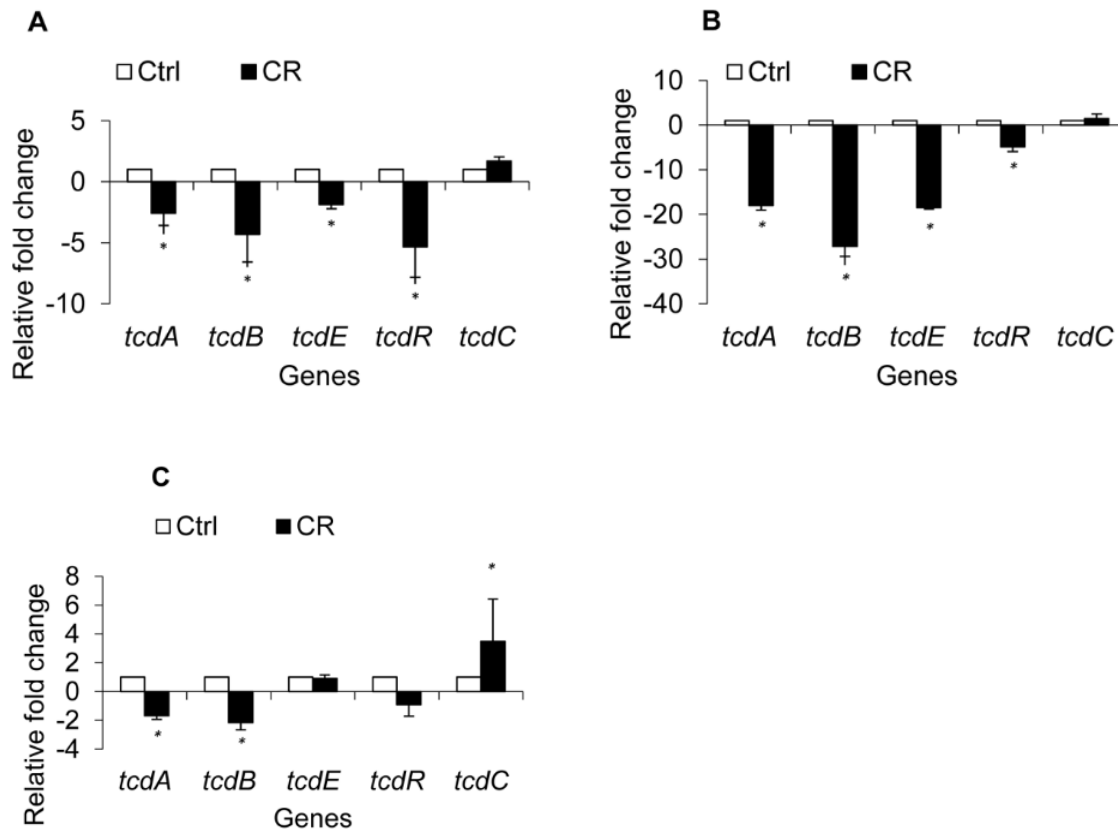


Figure 5. Effect of trans-cinnamaldehyde (TC) on *C. difficile* toxin regulatory genes.

Brain heart infusion with or without the sub-inhibitory concentration (SIC) of TC, (0.38 mM) was inoculated (10⁵ CFU/mL) separately with three hyper virulent *C. difficile* isolates, ATCC BAA 1870 (A); ATCC BAA 1053 (B) or ATCC BAA 1805 (C), and incubated anaerobically at 37 °C for 72 h. Bacterial pellets were harvested at 6 h and 12 h for RNA isolation and RT-qPCR for toxin regulatory genes. * Treatments significantly differed from the controls at 24 and 48 h of incubation ($p < 0.05$).

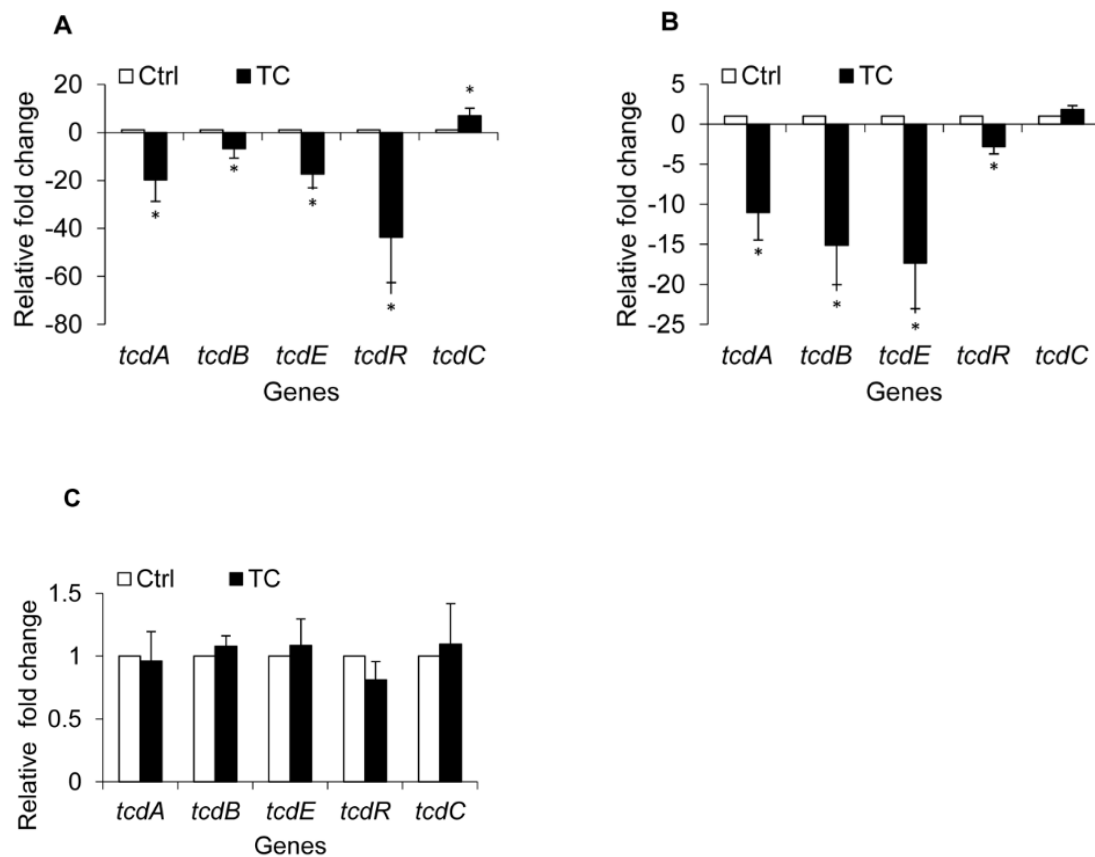


Figure 6. Effect of carvacrol (CR) and trans-cinnamaldehyde (TC) on *codY* mutant and wild type *C. difficile* toxin production.

Brain heart infusion with or without the sub-inhibitory concentration (SIC) of TC or CR, (0.38 and 0.60 mM respectively) was inoculated (10⁵ CFU/mL) separately with UK1 and LB-CD16 (*codY* mutant) *C. difficile* isolates, and incubated anaerobically at 37 °C for 24 h. The culture supernatants from groups TC, CR and Control (Ctrl) were collected at 24 of incubation for total toxin A and B quantitation by ELISA. * Treatments significantly differed from respective controls (p < 0.05).

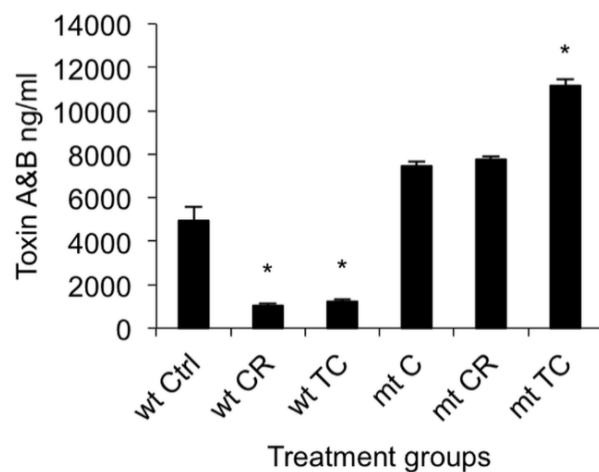
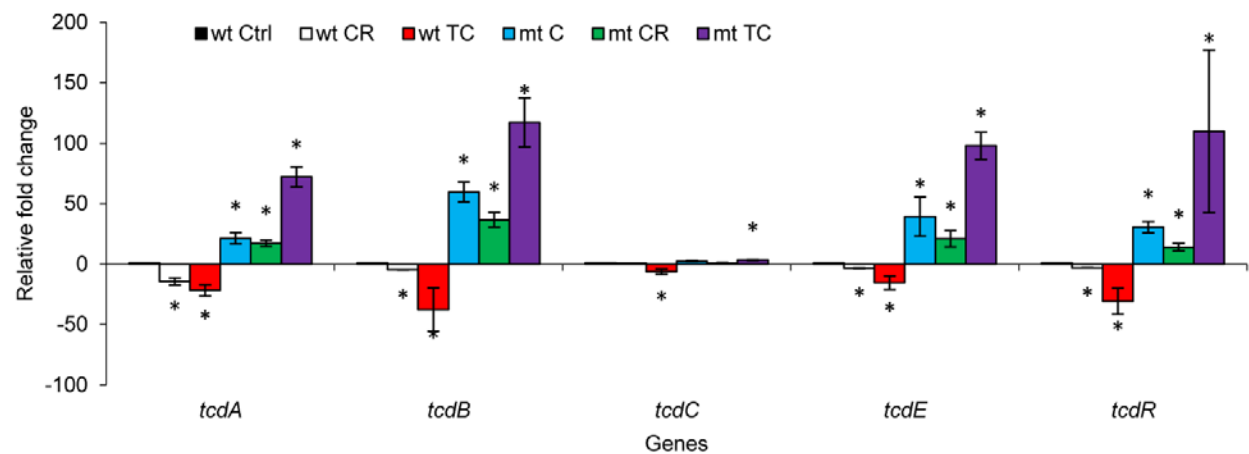


Figure 7. Effect of carvacrol (CR) and trans-cinnamaldehyde (TC) on *codY* mutant and wild type *C. difficile* toxin genes.

Brain heart infusion with or without the sub-inhibitory concentration (SIC) of TC or CR, (0.38 and 0.60 mM respectively) was inoculated (105 CFU/mL) separately with wild type (wt) UK-1 and its *codY* mutant (mt) *C. difficile* isolates, and incubated anaerobically at 37 °C for 72 h. Bacterial pellets were harvested at 6 h and 12 h for RNA isolation and RT-qPCR for toxin regulatory genes.

* Treatments significantly differed from respective controls ($p < 0.05$).



Chapter V

Effect of Carvacrol on *Clostridium difficile* sporulation and spore outgrowth

Abstract

Clostridium difficile is an anaerobic spore-forming pathogen that causes a serious toxin-mediated enteric disease in humans. Therapeutic agents that are capable of reducing *C. difficile* spore production could significantly minimize the transmission and relapse of *C. difficile* infections. This study investigated the ability of a food-grade, plant-derived compound, carvacrol (CR), to reduce *C. difficile* spore production, germination and spore outgrowth. Two hyper-virulent *C. difficile* isolates (ATCC BAA 1870 and 1805) were grown with or without the sub-inhibitory concentration (SIC) of CR. Total viable counts and heat-resistant spore counts were determined at different time intervals. Moreover, spores and vegetative cells were visualized using phase contrast microscopy. For determining the effect of CR on *C. difficile* germination and spore outgrowth, *C. difficile* spores were seeded in germination medium with or without the SIC and MIC of CR, and spore germination and spore outgrowth were measured by recording optical density at 600 nm. The effect of CR on *C. difficile* sporulation genes was also investigated using real-time qPCR. Carvacrol significantly reduced sporulation in *C. difficile* and down-regulated critical genes involved in spore production ($P < 0.05$). SIC or MIC of carvacrol did not inhibit *C. difficile* spore germination, however, MIC of the phytochemical completely inhibited spore outgrowth. Results suggest that CR could potentially be used to control the spread and relapse of *difficile* by reducing spore production and outgrowth.

Introduction

Clostridium difficile is a spore-forming, anaerobic bacterium that causes a toxin-mediated enteric disease in humans (Weese, 2010). *C. difficile* predominantly affects long-term hospital inpatients and the elderly undergoing prolonged antibiotic therapy (Hookman & Barkin, 2009). Prolonged antibiotic therapy results in the disruption of the normal enteric microflora, causing *C. difficile* spore germination and pathogen colonization in the intestine, with subsequent production of toxins (Dial et al., 2005). Although toxins are the major virulence factors responsible for the pathogenesis of *C. difficile* infection, spore formation, germination and spore outgrowth in *C. difficile* are critical components of CDAD transmission and relapse (Deakin et al., 2012b). *C. difficile* endospores can survive in the gut for a prolonged period of time (Paredes-Sabja et al., 2014c). This leads to germination, proliferation and colonization of *C. difficile* in the gut following prolonged antibiotic treatment that disrupts the normal gut flora, which in turn causes new infections or relapse of CDAD (Riggs et al., 2007; Sun et al., 2011b). Relapse of CDAD is reported in 25% of the patients undergoing anti-*C. difficile* therapy (Rupnik et al., 2009). Spore production in *C. difficile* is regulated by several genes, including *spo0A*, which is the master regulator of Spo0A, together with associated kinases and different sigma factors such as *sigH* (Rosenbusch et al., 2012; Saujet et al., 2011; Underwood et al., 2009). Highly resistant *C. difficile* spores shed in the faeces contaminate hospitals and healthcare facilities, which can cause infection through faeco-oral route or recurrence of CDAD in patients following germination and spore outgrowth (Barbut et al., 2009; Hookman & Barkin, 2009).

Despite the fact that exposure to broad spectrum antibiotics predisposes patients to CDAD by disrupting the normal gut flora (Bartlett, 1992; O'Connor et al., 2004), antibiotics still constitute the chief treatment strategy against *C. difficile*. In addition, the emergence of antibiotic resistance

in hypervirulent strains of *C. difficile* is documented (Prabaker & Weinstein, 2011; Spigaglia et al., 2011). Therefore, alternative therapeutic agents that can attenuate *C. difficile* virulence without disrupting the normal gut flora represent a viable control approach against the pathogen. Carvacrol (CR) is a monoterpenoid phenol present in oregano and thyme oils. Various pharmacological effects of CR, including antimicrobial and anti-inflammatory properties, have been demonstrated (Baser, 2008). Our previous study indicated that CR effectively inhibited *C. difficile* toxin production *in vitro*, without deleteriously affecting the major normal gastrointestinal flora in humans (Mooyottu et al., 2014e). The objective of this study was to investigate the potential of CR to inhibit spore production, spore germination and spore outgrowth *in vitro*, which are critical in the spread and relapse of *C. difficile* infection, and to delineate the potential mechanism(s) behind its effect.

2. Materials and methods

2.1 Bacterial strains and culture conditions

Two hypervirulent *C. difficile* isolates (ATCC BAA 1805, and 1870) were grown in brain heart infusion broth (BHI) supplemented with 5% yeast extract (Difco, Sparks, MD, USA) in a Whitley A35 anaerobic work station (Microbiology International, Frederick MD, USA) in the presence of 80% nitrogen, 10% carbon dioxide and 10% hydrogen at 37°C for 24 h. The bacterial population was determined by plating 0.1 ml portions of appropriate dilutions on BHI agar and *Clostridium difficile* moxalactam norfloxacin (CDMN) agar (Oxoid, Lenexa, KS, USA) supplemented with 5% horse blood, under strict anaerobic conditions at 37°C for 24 h. The cultures were sedimented by centrifugation (3600 g, 15 min, 4°C), and the pellet was washed twice, and resuspended in sterile phosphate-buffered saline (pH 7.3), and used as the inoculum.

2.2 *C. difficile* spore preparation

C. difficile spores were prepared as previously described, with slight modifications (Sorg & Dineen, 2009). Briefly, single colonies of ATCC BAA 1870 and 1805 were separately inoculated into BHIS broth (BHI plus cysteine) and cultured overnight at 37°C under anaerobic conditions. A volume of 150 µl aliquot of overnight culture was spread onto BHI agar (Oxoid) in six well plates and cultured anaerobically for 10 days at 37°C in a Whitley A35 anaerobic workstation to allow sporulation. After 10 days, spores were harvested from the wells by flooding with 2 ml of ice cold sterile water. The spore suspension was heat-treated at 60°C for 20 min to kill any vegetative cells, and washed six times in dH₂O by centrifuging at 16,000 g for 5 min. Spore suspensions were examined for purity by phase-contrast microscopy before storage at -20 °C prior to use.

2.3 Carvacrol and determination of SIC and MIC on *C. difficile*

The sub-inhibitory concentration (SIC) of CR (≥98% purity, Sigma Aldrich, St. Louis, MO, USA) against *C. difficile* was determined, as previously described (Johnny et al., 2010). Approximately 5.0 log CFU *C. difficile* (ATCC BAA 1870 and 1805) was inoculated separately in tissue culture plates containing 2 ml of BHI, followed by the addition of 1 to 10 µl of CR with an increment of 0.5 µl. The plates were then incubated in an anaerobic chamber incubator at 37°C for 24 h with 80% N₂, 10% H₂ and 10% CO₂, and bacterial growth was monitored by determining optical density at 600 nm (OD₆₀₀). The lowest concentration of CR that inhibited bacterial growth was selected as the MIC, and the two highest CR concentrations below the MIC that did not inhibit bacterial growth after 24 h of incubation were selected as the SICs for this study.

2.4 Effect of CR on *C. difficile* growth and sporulation

The effect of CR on *C. difficile* growth and sporulation was determined according to a previously published protocol (Babakhani et al., 2012). Briefly, BHI with or without the SICs of

CR was inoculated (10^5 CFU/ml) separately with each *C. difficile* isolate, and incubated at 37°C for 96 h anaerobically as before. Samples were withdrawn at different time intervals (24, 48, 72 and 96 h) for quantitation of heat-resistant spores (survivors of incubation at 60°C for 20 min) and total viable count (TVC) by serially diluting each sample in PBS and plating on BHIS agar supplemented with 0.1% taurocholate. In addition, sporulation in BHIS at the specified time intervals was visualized under phase contrast microscopy (Burns et al., 2011). A 10 µL aliquot of the culture from different treatment groups were loaded onto a microscopy slide, air-dried, and visualised under 1.5×40x magnification.

2.5 Effect of SIC and MIC of CR on *C. difficile* spore germination and outgrowth

To study the effect of CR on *C. difficile* spore germination, 100 µl suspension containing 10^5 spores/ml was added to the wells of a 12-well plate containing 1.9 ml of pre-warmed, pre-reduced BHI supplemented with 0.1% sodium taurocholate (Sigma-Aldrich) inside an anaerobic workstation. The plates were closed inside the workstation with lids and sealed with a sealant. Brain heart infusion without taurocholate and spore suspensions or medium replaced with dH₂O were included as controls. A well with resazurin 0.1 mg/ml was included for examining anaerobiosis in the plates during reading. Optical density at 600 nm was recorded using Synergy plate reader (Biotek, Winooski, VT, USA) at 37°C over a 24 h time period, with readings taken at 10 min intervals, and was expressed as a percentage of the initial OD₆₀₀ (t/t_0). Spore germination was measured as the initial loss of OD₆₀₀ and spore outgrowth was measured by recording the increase in OD₆₀₀ followed by spore germination, as described previously (Allen et al., 2013; Paredes-Sabja et al., 2008).

2.6. Real-time Quantitative PCR (RT-qPCR)

To determine the effect of CR on *C. difficile* genes involved in spore production, total RNA was isolated from early stationary phase cultures grown with and without the SIC of CR (Blossom & McDonald, 2007). The culture supernatant was harvested by centrifugation at $3000 \times g$ for 10 min at 4°C. The bacterial pellet was resuspended in RNawiz solution (Ambion, Austin, TX, USA), flash frozen in liquid nitrogen, and stored at -80°C. Total RNA extraction was performed using the Ambion RiboPure Bacteria RNA kit (Ambion) according to the manufacturer's instructions, followed by DNase I digestion using Turbo DNase I (Ambion). The RNA obtained after each DNase I digestion was purified further using the Qiagen RNeasy RNA column purification kit (Qiagen, Germantown, MD, USA). The cDNA was synthesized using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). RT-qPCR analysis of the genes associated with spore production was performed using published primers for genes associated with sporulation (Babakhani et al., 2012; Saujet et al., 2011) and normalized against 16S rRNA gene expression. Twenty-five µl reactions were performed in triplicate using iTaq SYBR (Bio-Rad, Hercules, CA, USA). The relative fold change in gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001).

2.7 Statistical Analysis

All experiments had duplicate samples and the study was repeated three times. The data were analyzed using the one-way ANOVA. Differences between means were considered significantly different at $p < 0.05$.

3. Results

3.1 Sub-inhibitory concentrations of CR

The MIC of CR against *C. difficile* was 1.2 mM. The two highest SICs of CR were found to be 0.9 mM and 0.6 mM. These two concentrations of CR did not inhibit the growth of *C. difficile* isolates after 24 h incubation at 37°C.

3.2 Effect of CR on *C. difficile* growth and sporulation

The effect of SICs of CR on *C. difficile* spore production is shown in Fig. 1A and 1B. In both *C. difficile* isolates, a significant decrease in spore counts was observed after 48 and 72 h of incubation in CR-treated samples compared to control ($p < 0.05$). In *C. difficile* BAA 1870, CR at 0.6 mM and 0.9 mM resulted in ~ 1.5 log CFU/ml and 2.0 log CFU/ml reduction in spore counts at 48 and 72 h of incubation, respectively. At 96 h, although spore counts in 0.6 mM CR-treated samples grew back by ~ 1.0 log CFU/ml, CR treatment at 0.9 mM reduced the spore count ~ 3 log CFU/ml (Fig. 1A). However, differences in total viable counts (plated without heat treatment) between CR-treated samples and untreated control were minimal ($p > 0.05$). Similar results were also observed in *C. difficile* BAA 1805 (Fig. 1B), where ~ 1 log CFU/ml reduction in spore count was observed in 0.6 mM CR treated cultures at 48, 72 and 96 h. Similarly, in the presence of 0.9 mM CR, a 2 log reduction in spore counts was observed consistently from 48 h through 96 h. Moreover, the differences in total viable counts between CR-treated and control samples were less than 1.0 log CFU/ml throughout the sampling times ($p > 0.05$). The results from the sporulation experiments were consistent with phase contrast microscopy results of CR-treated and untreated *C. difficile* cultures (Fig. 2). In untreated control, more phase bright spores and a fewer vegetative bacteria populations were observed throughout the microscopic field. Conversely, in CR-treated *C. difficile* culture, more number of vegetative bacteria and a lesser phase bright spores and a few phase dark spores were observed after 48 h of incubation.

3.3 Effect of CR on *C. difficile* spore germination and outgrowth

The effect of SIC (0.6 mM) and MIC (1.2 mM) of CR on *C. difficile* spore germination and outgrowth over a 24 h time period was determined. In *C. difficile* BAA 1870, spore outgrowth was observed in untreated wells, as indicated by an increase in OD₆₀₀ starting at 11 h, with continued increase over the 24 h duration (Fig. 3A). However, in the presence of 0.6 mM CR, *C. difficile* spore outgrowth was delayed, as indicated by a lower absorbance at each sampling time point ($p < 0.05$). In addition, the MIC of CR (1.2 mM) completely inhibited spore outgrowth, as evident from a lack of increase in absorbance at 600 nm. In *C. difficile* BAA 1805, CR was found more effective, where at 0.6 mM spore outgrowth was delayed by 3h and the growth was found to be decreased throughout the sampling points ($p > 0.05$) (Fig. 3B). Moreover, CR at 1.2 mM completely shut down spore outgrowth as observed with *C. difficile* BAA 1870. No significant difference was observed in the initial dip in the OD₆₀₀ in both CR treated (SIC and MIC) and untreated *C. difficile*, indicating that, neither SIC nor MIC of CR was able to reduce *C. difficile* spore germination.

3.4 Effect of CR on *C. difficile* spore production genes

To investigate if the observed reduction in *C. difficile* spore production was due to a modulation in the expression of genes known to be involved in sporulation, RT-qPCR was performed. The results showed that CR significantly down-regulated the transcription of *C. difficile* sporulation genes, where the expression of *spo0A* was down-regulated by 22 folds in BAA 1870 and 13 folds in BAA 1805, and that of *spoIIA* by 23 and 25 folds in BAA 1870 and BAA 1805, respectively. (Fig. 4A and 4B). In addition, the transcription of *spoIIR* was decreased by 7 and 8 folds and that of *spoIIID* by 5 and 7 folds in *C. difficile* BAA 1870 and 1805, respectively ($p < 0.05$). Similarly, *sigH* was down-regulated by 19 and 26 folds in *C. difficile* BAA 1870 and

BAA 1805,s respectively ($p < 0.05$). Likewise, the expression of *CD2492* was also reduced by 14 and 7 folds in *C. difficile* BAA 1870 and BAA 1805 isolates, respectively ($p < 0.05$).

4. Discussion

C. difficile is a major nosocomial pathogen, and recent epidemiologic data have shown that *C. difficile* has surpassed methicillin-resistant *Staphylococcus aureus* as the most commonly acquired hospital infection (Miller et al., 2010). Sporulation is a critical virulence factor in *C. difficile* pathogenesis. *C. difficile* spores being survival structures that can withstand unfavorable physical, chemical and metabolic conditions, play an important role in bacterial persistence in the environment, transmission to new susceptible individuals and relapse in temporarily recovered patients (Paredes-Sabja et al., 2014c). *C. difficile* spores enter the host by feco-oral route, germinate in the presence of bile acid in the small intestine and colonize the colon, where vegetative cells produce toxins, leading to severe diarrhea (Hookman & Barkin, 2009). Further, sporulation of vegetative cells occurs in the colon, and newly formed spores shed in the feces can potentially contaminate hospitals and healthcare facilities leading to new infections (Simor et al., 2002). Therefore, agents reducing *C. difficile* sporulation and spore outgrowth in the human gastrointestinal tract could effectively control *C. difficile* infection, transmission, and relapse (Johnson, 2009; Maroo & Lamont, 2006). Results from this study suggest that CR, a plant-derived, food-grade compound which has been previously shown to exert anti-toxigenic effect in *C. difficile* can significantly reduce *C. difficile* spore production and spore outgrowth *in vitro*.

Results from this study demonstrate that the SICs of CR significantly reduced spore production by *C. difficile* at 48, 72 and 96 h of incubation ($p < 0.05$). The reduction in sporulation in CR-treated samples was determined by dilution and plating method, and also visualized using phase contrast microscopy. Interestingly, no reduction in total number of viable cells (TVC, which

constitute both vegetative cells and spores) was observed in CR-treated *C. difficile* cultures compared to untreated control. These results indicate that CR was able to specifically inhibit the formation of spores from the vegetative cells in broth culture. A slight increase in TVC of CR-treated cultures could be due to the presence of higher proportion of vegetative cells, which have superior detection limit on plating compared to spores. Since the SICs of CR were used in the sporulation experiments, the decrease observed in *C. difficile* spore production in CR-treated samples was not due to inhibition of bacterial growth by CR, but could be attributed to their potential ability to reduce the transcription of virulence genes associated with spore production. Concurring this, the gene expression studies indicated that CR significantly down-regulated critical genes responsible for *C. difficile* sporulation. In the germination experiments, the results revealed that the SIC of CR (0.6 mM) significantly reduced *C. difficile* spore outgrowth (outgrowth of vegetative cells from the newly germinated spores) compared to controls ($p < 0.05$), whereas CR at the MIC (1.2 mM) completely inhibited the spore outgrowth. These results suggest that vegetative cells from newly germinated spores are more sensitive to CR compared to vegetative *C. difficile* cells grown in broth.

A potential alternate strategy for controlling microbial infections is the use of anti-virulence drugs, which target reducing bacterial virulence rather than inhibiting bacterial growth (Rasko & Sperandio, 2010), thereby presenting a lesser selective pressure for development of bacterial antimicrobial resistance (Cegelski et al., 2008; Hughes & Sperandio, 2008; Hung et al., 2005). The results of the current study indicate that CR inhibited sporulation in *C. difficile*, which aids in bacterial virulence especially towards CDAD transmission and relapse. Moreover, prior research conducted in our laboratory revealed that SICs of CR inhibited the production of *C. difficile* toxins, which represent another important virulence factor in the pathogen without

inhibiting the normal gut bacteria (Mooyottu et al., 2014b). Research by others have also reported that CR exerted no adverse effects on endogenous bacterial populations, including *Lactobacilli* and *Bifidobacteria* in pigs and poultry (Jamroz et al., 2005). To conclude, the results from this study suggest the potential of CR for controlling *C. difficile* by reducing spore production and outgrowth, thereby warrants follow-up *in vivo* studies to confirm the findings.

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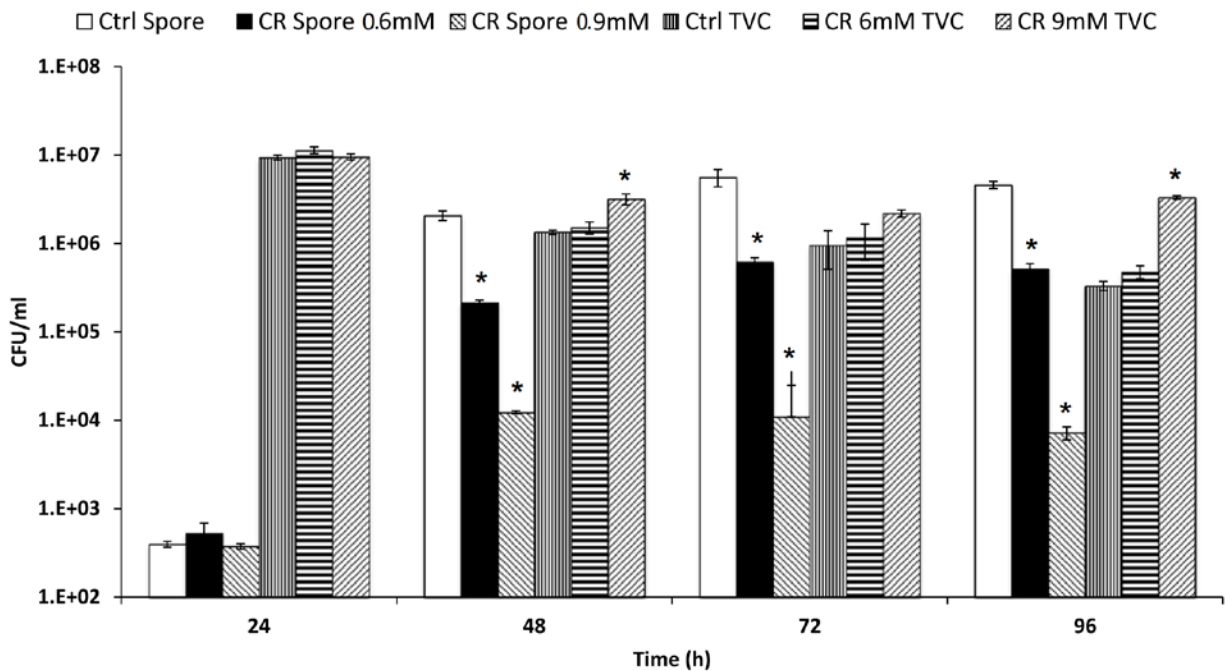
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Figures

Fig. 1 Effect of carvacrol (CR) on *C. difficile* BAA 1870 (1A) and 1805 (1B) growth and sporulation

Brain heart infusion with or without the Sub-inhibitory concentrations (SIC) of CR, (0.6 mM and 0.9 mM) was inoculated (10^5 CFU/ml) separately with *C. difficile* BAA 1870 (A) and BAA 1805 (B), and incubated anaerobically at 37°C for 96 h. Samples were withdrawn at different times for quantitation of heat-resistant spores (survivors of incubation at 60°C for 20 minutes) and total viable count (TVC) by serially diluting each sample in PBS and plating each in duplicate on BHIS agar supplemented with 0.1% taurocholate. * Treatments significantly differed from the respective controls ($p < 0.05$)

1A



1B

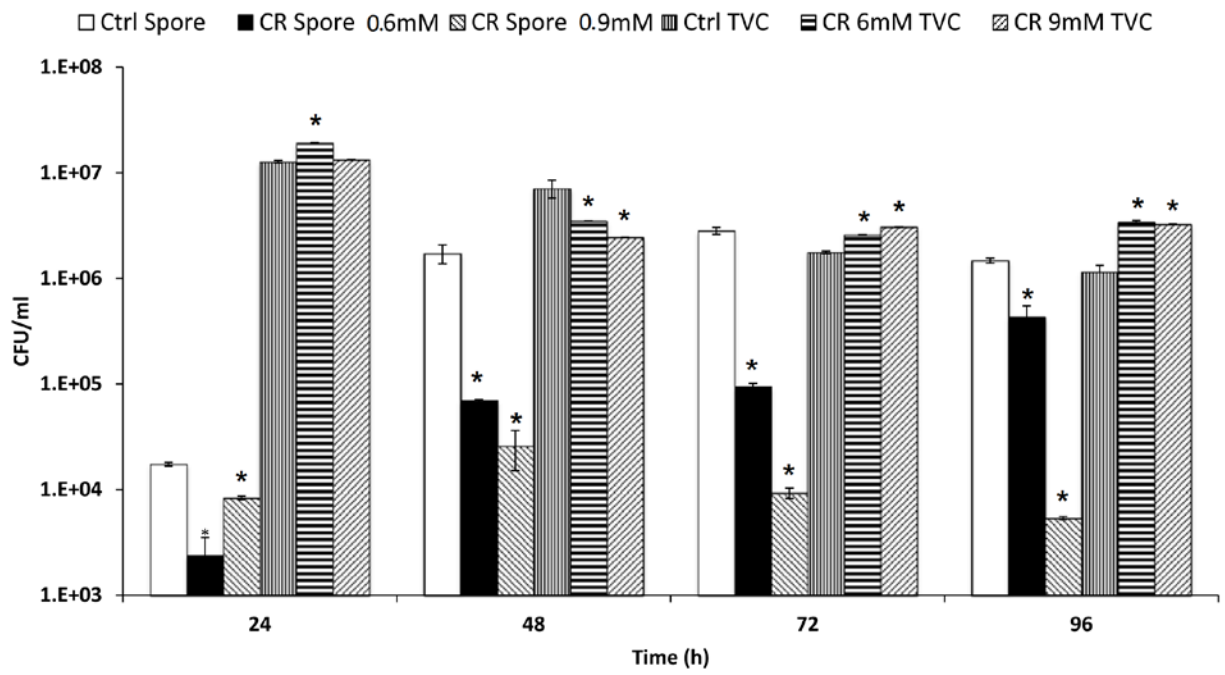


Fig. 2: Effect of carvacrol (CR) on *C. difficile* ATCC BAA 1870 and BAA 1805 sporulation.

Brain heart infusion with or without the Sub-inhibitory concentration (SIC) of CR, (0.6 mM) was inoculated (10^5 CFU/ml) separately with *C. difficile* BAA 1870 and BAA 1805, and incubated anaerobically at 37°C for 96 h, Sporulation at 48 h of incubation in BHIS was visualized under phase contrast microscopy. A 10 μ l aliquot of the culture from different treatment groups were loaded onto a microscopy slide, air dried, and visualised at 1.5 \times 40x magnification. A) Control *C. difficile* BAA 1870 B) *C. difficile* BAA 1870 with 6mM CR, C) Control *C. difficile* BAA 1805, D) *C. difficile* BAA 1805 with 6mM CR.

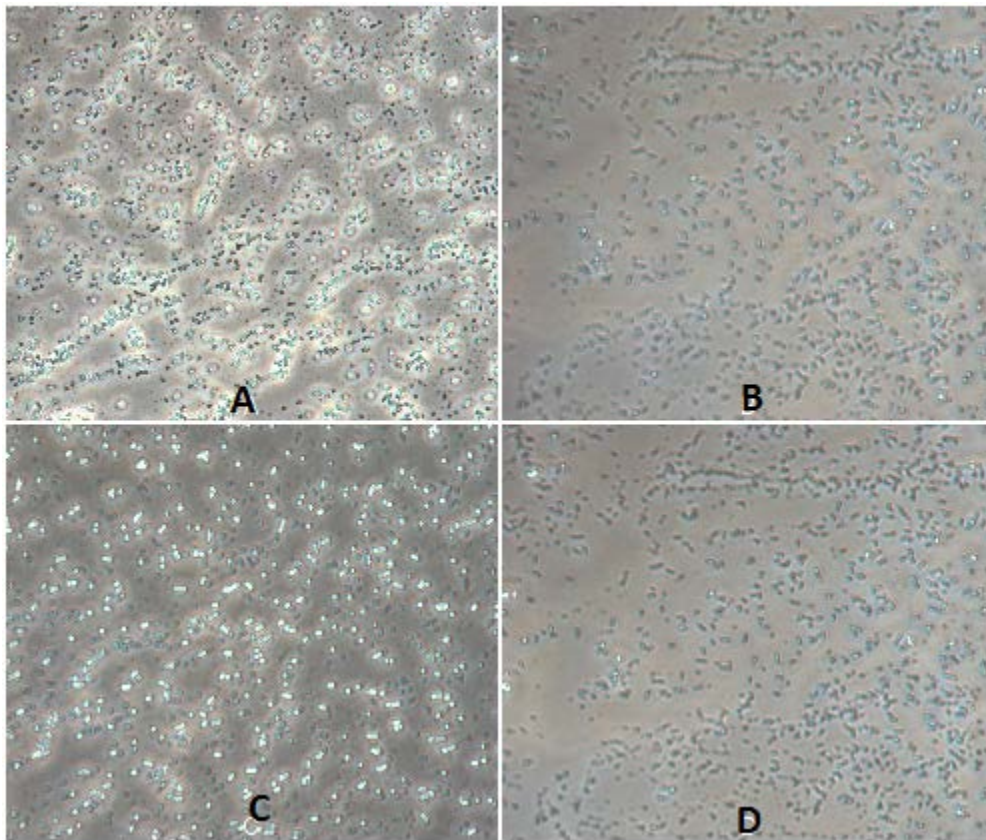
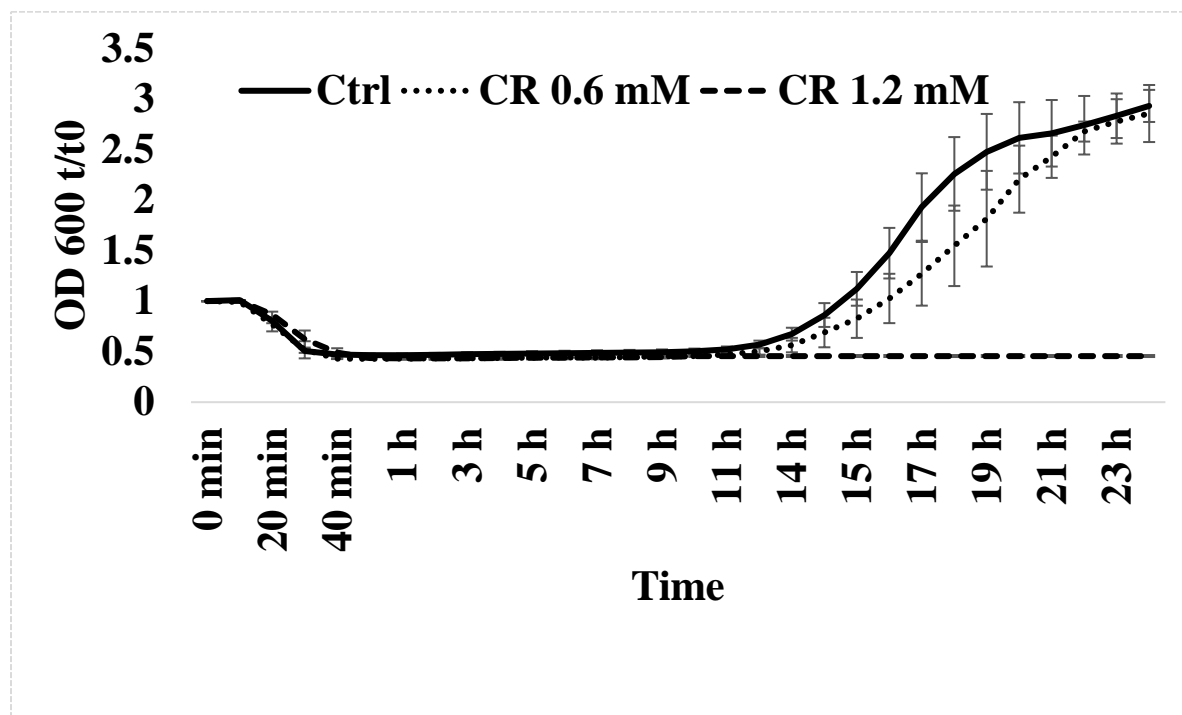


Fig. 3: Effect of carvacrol (CR) on germination and outgrowth of *C. difficile* BAA 1870 (3A) and 1805 (3B) spores.

Spore suspension containing 10^5 spores/ml was added to the wells of a 12-well plate containing 1.9 ml of pre-warmed, pre-reduced BHI supplemented with 0.1% sodium taurocholate was incubated with 0mM, 6mM or 12Mm CR inside an anaerobic workstation. The plates were closed inside the workstation with lids and sealed with a sealant. BHI media without taurocholate, and spore suspensions or culture media replaced with dH₂O were included as controls. A well with resazurin 0.1 mg/ml was included for examining anaerobiasis in 12 well plate during reading. Optical density at 600 nm (OD600) were recorded using Synergy plate reader at 37 °C over a 24 h time period with readings taken at 10 min intervals and was expressed as a percentage of the initial OD600 (t/t_0). Germination was measured as the initial loss of OD600 and Spore outgrowth was measured by recording the increase in OD600 followed by spore germination.

3A



3B

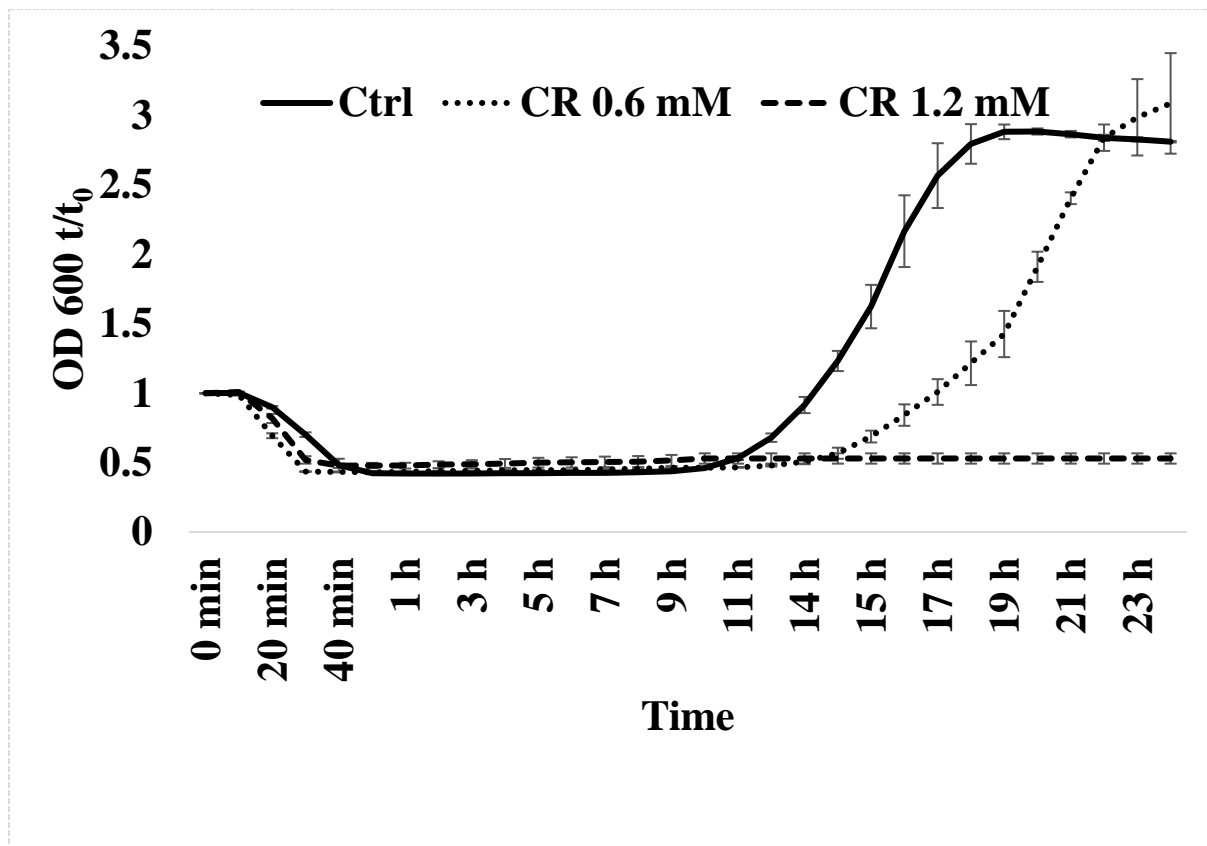
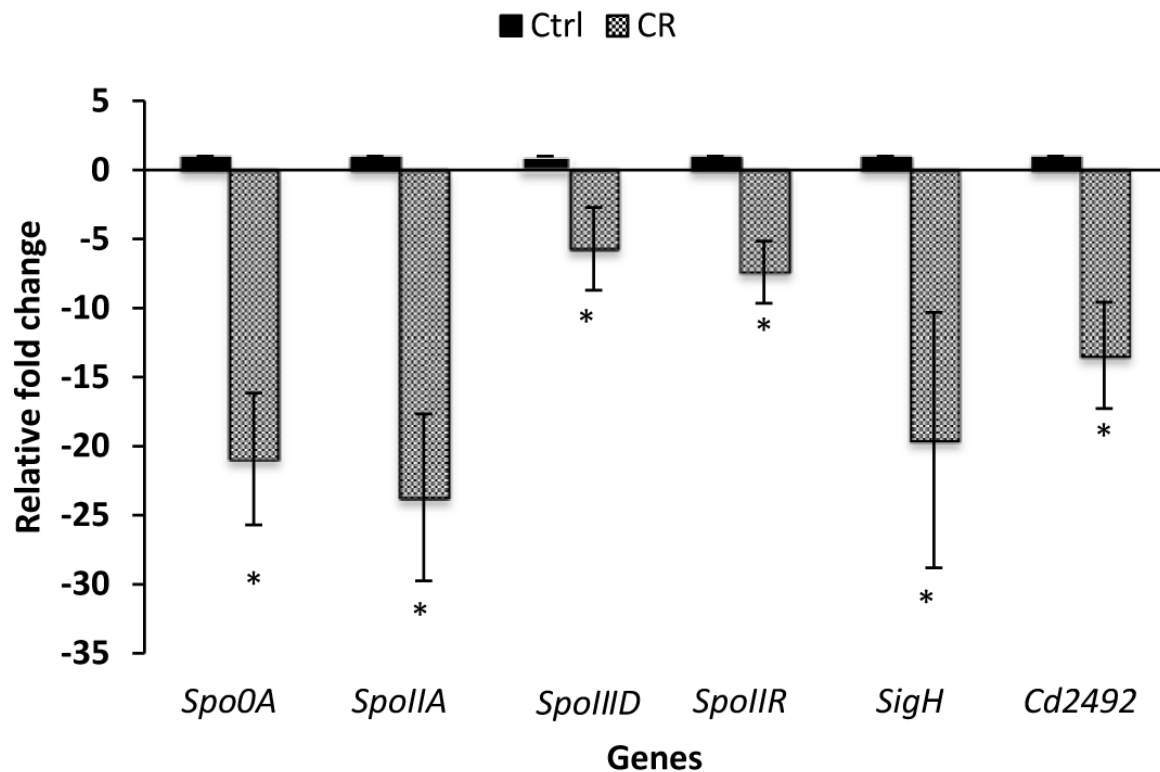


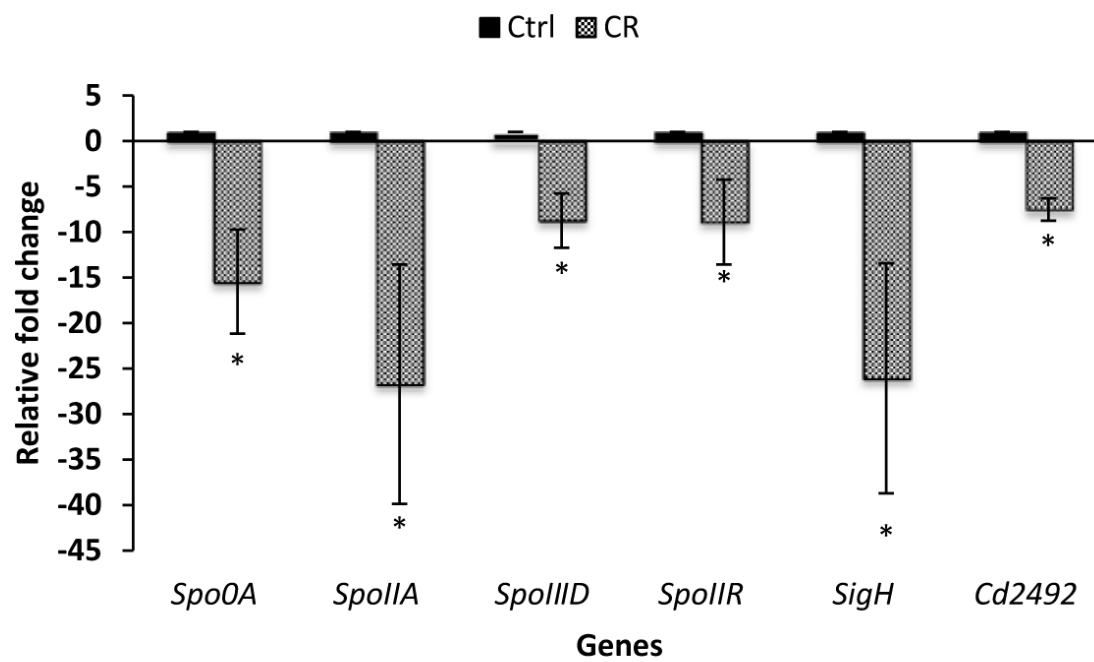
Fig. 4: Effect of carvacrol (CR) on *C. difficile* ATCC BAA 1870 (4A) and 1805 (4B) toxin regulatory genes.

Brain heart infusion with or without the sub-inhibitory concentration (SIC) of CR, (0.6 mM) was inoculated (10^5 CFU/ml) separately with *C. difficile* isolates, BAA 1870 (A), BAA or BAA 1805 (B), and incubated anaerobically at 37°C for 72 h. Bacterial pellet was harvested at 12 h for RNA isolation and RT-qPCR for spore production genes. * Treatments significantly differed from the controls at 24 and 48 h of incubation ($p < 0.05$).

4A



4B



Chapter VI

Protective Effect of Carvacrol against Gut Dysbiosis and *Clostridium difficile* Associated Disease in a Mouse Model

Abstract

Clostridium difficile is an anaerobic sporeforming pathogen causing a toxin-mediated enteric disease in humans. *C. difficile* predominantly affects hospital inpatients undergoing prolonged antibiotic therapy, which results in enteric dysbiosis, leading to *C. difficile* spore germination, pathogen colonization in the intestine and subsequent toxin production. Therapeutic agents that inhibit *C. difficile* without causing enteric dysbiosis could improve the clinical outcome of *C. difficile* infections. Our previous studies indicated that carvacrol (CR), a plant-derived compound significantly inhibited *C. difficile* toxin production and spore outgrowth *in vitro*. This study investigated the effect of CR on antibiotic-associated gut dysbiosis and *C. difficile* infection in a mouse model.

Five to six-week-old C57BL/6 mice were randomly divided into seven treatment groups (challenge and control) of eight mice each. Mice were fed with irradiated feed supplemented with CR (0%, 0.05%, and 0.1%); the challenge groups were made susceptible to *C. difficile* by orally administering an antibiotic cocktail in water and an intra-peritoneal injection of clindamycin. Both challenge and control groups were infected with 10^5 CFU/ml of hypervirulent *C. difficile* (ATCC 1870) spores or PBS, and observed for clinical signs for ten days. Respective control groups for CR, antibiotics, and their combination were included for investigating their effect on mouse enteric microflora. Mouse body weight and clinical and diarrhea scores were recorded daily post infection. Fecal samples were collected for microbiome analysis using rRNA sequencing in MiSeq platform.

Carvacrol supplementation significantly reduced the incidence of diarrhea and improved the clinical and diarrhea scores in mice ($p < 0.05$). Microbiome analysis revealed a significant increase in Proteobacteria and reduction in the abundance of protective bacterial flora in antibiotic-treated and *C. difficile*-infected mice compared to controls ($p < 0.05$). However, CR

supplementation positively altered the microbiome composition, as revealed by an increased abundance of beneficial bacteria, including Firmicutes, and significantly reduced the proportion of detrimental flora such as Proteobacteria, without significantly affecting the gut microbiome diversity compared to control. Results suggest that CR could potentially be used to control gut dysbiosis and reduce *C. difficile* infection.

1. Introduction

Clostridium difficile infection is the major cause of antibiotic-associated diarrhea in hospital settings around the world (Hookman & Barkin, 2009; McFarland, 2008). *C. difficile* principally causes a serious toxin-mediated colitis in the elderly and immunocompromised patients (Weese, 2010). Annually, more than 300,000 cases of *C. difficile* associated diseases (CDAD) are reported in the United States, resulting in more than US\$3 billion as health care costs (Ghose et al., 2007; Wilkins & Lyster, 2003). A recently emerged, highly toxigenic and hyper-virulent *C. difficile* strain NAP1/ribotype 027 has been implicated in increasing incidence of CDAD among patients all over the world (Blossom & McDonald, 2007; Hookman & Barkin, 2009; Sunenshine & McDonald, 2006).

C. difficile infection has been associated with the use of antibiotics and gastric acid suppressing agents that result in gut dysbiosis (Bartlett, 1992; Dial et al., 2005; Kelly & LaMont, 1998). Prolonged antibiotic therapy results in the disruption of the normal enteric microflora, leading to an altered microbial composition such as increased population of Proteobacteria and reduced proportion of Bacteroides and Firmicutes in the gut microbiome (Ling et al., 2014; Seekatz & Young, 2014; Shahinas et al., 2012; Theriot et al., 2014). Consequently, gut dysbiosis results in the germination of spores and selection for *C. difficile* in the intestine. Following spore germination and outgrowth in the presence of a disrupted gut flora, the vegetative cells of *C. difficile* produce potent toxins known as toxin A and toxin B (Voth & Ballard, 2005). *Clostridium difficile* toxins (A and B) are functionally glucosyl transferases, which inactivate the Rho family GTPases associated with F-actin regulation, and cause disruption of the cytoskeleton and intestinal epithelial tight junctions (Keel & Songer, 2006; von Eichel-Streiber et al., 1999). This leads to a severe inflammatory response with the release of cytokines and leukotrienes, causing pseudomembrane

formation and severe diarrhea (Hookman & Barkin, 2009; McDonald et al., 2006; Sunenshine & McDonald, 2006). Since gut dysbiosis is considered as the most important predisposing factor in CDAD, emerging and novel therapeutic approaches, including fecal microbiome transplantation (FMT) primarily aimed at restoration of the normal gut flora in CDAD patients are explored (Kassam et al., 2013).

Despite the fact that a majority of the currently used antibiotics can predispose CDAD by disrupting the normal gut flora, antibiotics are still used as the primary line of treatment against infection (Bartlett, 1992; O'Connor et al., 2004). Nonetheless, many of the anti- *C. difficile* antibiotics are found to predispose CDAD in patients by inducing gut dysbiosis (McFarland, 2008; O'Connor et al., 2004; Shah et al., 2010). Moreover, the emergence of antibiotic resistant strains of hypervirulent *C. difficile* is documented worldwide (Spigaglia et al., 2011; Steiner et al., 2012). The Centers for Disease Control and Prevention (CDC) recently listed *C. difficile* as one among the three urgent threats in their report on emerging pathogens with antibiotic resistance (Steiner et al., 2012). Since the toxins are the major virulence factors for CDAD, a search for alternative therapeutic agents, which can reduce *C. difficile* virulence without affecting normal gastrointestinal flora opens a new research area.

Carvacrol (CR) is a food grade, monoterpenoid phytophenol that is naturally present in oregano and thyme oil. Diverse pharmacological actions of carvacrol, including antimicrobial and anti-inflammatory activities have been previously demonstrated (Baser, 2008). A recent study from our laboratory suggested the potential use of CR as an anti-*C. difficile* therapeutic agent due to its inhibitory effect on *C. difficile* toxin production without affecting the growth of beneficial gut bacteria *in vitro* (Mooyottu et al., 2014c). This study demonstrated that CR significantly inhibited toxin production in hypervirulent *C. difficile* strains by modulating toxin production

genes. Therefore, this study investigated the therapeutic effect of CR against *C. difficile* in an *in vivo* model, specifically its impact on the clinical course of *C. difficile* infection and the host microbiome. Mouse is a well-established model of *C. difficile* infection (Chen et al., 2008; Sun et al., 2011a), and antibiotic-associated *C. difficile* infection can be induced in a mouse model by administering antibiotics orally and intraperitoneally, followed by inoculation of *C. difficile* spores (Chen et al., 2008; Sun et al., 2011a).

2. Materials and methods

2.1 Ethics statement, animals, and housing

This study was conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Connecticut. All recommended guidelines for the care and use of animals were followed. Six-week-old C57BL/6 mice were obtained from Charles River (Boston, MA). Animals were housed in a biohazard level II, AALAC-accredited facility and monitored twice daily for health. Mice were provided with autoclaved food, water, and bedding, with 12-h light/dark cycles. All cage changes, *C. difficile* spore infection, and sample collections were performed under a laminar flow hood using proper personal protective equipment. The work area was sterilized using 10% bleach between experimental treatment groups to prevent cross-contamination. The mice were housed in pairs in a cage, and four cages were included for each treatment in each of the experiments.

2.2 Mouse model of *C. difficile* infection and treatment groups

The infection model adopted for this study is a modification of the method described by Chen et al. (2008). Five to six-week-old female mice were randomly divided into eight treatment groups of eight animals each (Table 1). The animals were subjected to food restriction for 12 h, and given powdered feed supplemented with 0, 0.05 and 0.10% of CR. After 7 days, an antibiotic

mixture was added in drinking water (kanamycin, 0.4 mg/mL, gentamicin, 0.03 mg/mL, colistin, 850 U/mL, metronidazole, 0.215 mg/mL, and vancomycin, 0.045 mg/mL) for 3 days. After the antibiotic treatment, all mice were given regular autoclaved water for two days, and all animals, including the control group received a single dose of clindamycin (10 mg/kg, maximum volume of injection 0.5 ml/mouse using a 27 gauge gavage needle and syringe) intraperitoneally one day before *C. difficile* challenge. This antibiotic pre-treatment was intended to disrupt the normal gut flora of mice and facilitate *C. difficile* colonization. All animals were infected by oral gavage with 10^5 colony-forming units (CFU) per 0.1 ml total volume of hypervirulent *C. difficile* spores (ATCC BAA 1805) using a straight 18-gauge needle with 1" shaft length, and were monitored for signs of CDAD such as diarrhea, hunched posture and wet tail using a mouse clinical score sheet (Tables 1 & 2). Animals were observed twice daily for ten days for mortality and morbidity. The individual weight of each animal was measured every day. Fecal samples from all animals were collected on alternate days post infection. The animals were euthanized at the end of the experiment (10th day after *C. difficile* challenge).

2.3 DNA extraction, PCR amplification, and sequencing of taxonomic marker:

DNA was extracted from 0.25g of fecal sample (collected on the 2nd day post inoculation, DPI) from all treatment groups using the MoBio PowerMag Soil 96 well kit (MoBio Laboratories, Inc), according to the manufacturer's protocol for the Eppendorf ep Motion liquid handling robot. DNA quantification was performed using the Quant-iT PicoGreen kit (Invitrogen, ThermoFisher Scientific). Partial bacterial 16S rRNA genes (V4) were amplified using 30 ng extracted DNA as template. The V4 region was amplified using 515F and 806R with Illumina adapters and dual indices (8 basepair golay on 3'; Caporaso, 2012), and eight basepair on the 5' (Kozich, 2013). Samples were amplified in triplicate using Phusion High-Fidelity PCR master mix (New England

BioLabs) with the addition of 10 µg BSA (New England BioLabs). The PCR reaction was incubated at 95°C for 3.5 minutes, then 30 cycles of 30 s at 95.0°C, 30 s at 50.0°C and 90 s at 72.0°C, followed by a final extension at 72.0°C for 10 minutes were used. The PCR products were pooled for quantification and visualization using the QIAxcel DNA Fast Analysis (Qiagen). The PCR products were normalized based on the concentration of DNA from 250-400 bp and pooled using the QIAgility liquid handling robot. The pooled PCR products were cleaned using the Gene Read Size Selection kit (Qiagen) according to the manufacturer's protocol. The cleaned pool was sequenced on MiSeq using v2 2x250 base pair kit (Illumina, Inc).

2.4 Sequence Analysis

The microbiome analysis was set up as a completely randomized design with treatments done in replicates of six. The sequences were filtered and clustered using Mothur 1.36.1 based on a published protocol with slight modifications (Kozich et al., 2013). Operational taxonomic units (OTUs) were clustered at 97% sequence similarity. Downstream analysis of samples was done using R version 3.2. The alpha diversity was calculated using inverse Simpson to measure the richness and evenness of the OTUs. The effect of treatment on the alpha diversity was analyzed using Tukey's Test. A permutational multivariate analysis (PERMANOVA, adonis function, 75 permutations) was conducted to analyze the effect of various treatments on the bacterial community composition. Significant change in alpha diversity was determined by Anova followed by Tukey's honest significant differences adjusting for multiple comparisons (p-value=0.05). NMS ordinations were run in R (v 3.3.0) using metaMDS in the vegan (v 2.3-5) package after calculating the stress scree plots to determine the number of axes required to achieve stress below 0.2, plotted using ggplot2 (v 2.1.0). Finally, the relative abundance of OTUs of major phyla, order,

and genera was determined to assess the effect of treatment. Tukey's Test was used to identify changes in groups of bacteria based on treatment and the significance was detected at $P < 0.05$.

2.5 Statistical analysis

The results were expressed as means \pm standard errors of the means (SEM). The differences between the experimental groups were compared using the analysis of variance (ANOVA). Two-way ANOVA was used to compare experimental groups across the days. The differences between two groups were analyzed using unpaired Student's *t*-test. The statistical significance level was set at a *P* value of <0.05 .

3. Results

3.1 Effect of CR supplementation on the incidence of diarrhea and severity of *C. difficile* infection in mice

In order to assess the prophylactic effect of CR against *C. difficile* associated diarrhea in mice, the animal diets were supplemented with CR at two different concentrations (0.05% and 0.1%) in feed prior to antibiotic treatment and subsequent *C. difficile* infection (Ant + CD + 0.05% CR and Ant + CD + 0.1% CR). The oral administration of 10^5 CFU/ml NAP1 *C. difficile* spores resulted in high morbidity and low mortality in infected mice. In *C. difficile* infected control groups (Ant+CD), 75% of the animals showed severe diarrhea on 1DPI, and 90% of the animals showed severe diarrhea on 2DPI. Two animals died on 1DPI, and no further mortality was observed in this group. No increase in the incidence of diarrhea was observed after 2DPI (Fig. 1). Interestingly, only 50% of the animals showed diarrhea in 1DPI in Ant + CD + 0.05% CR group (animals supplemented with 0.05% CR prior to the antibiotic treatment and subsequent *C. difficile* infection), no increase in the incidence of diarrhea was reported after 1DPI. In Ant + CD + 0.1% CR group, only 10% of the animals showed diarrhea on 1DPI, which further increased to 25% on

2DPI and no further incidents were observed on subsequent days. In both CR-treated and *C. difficile* infected groups (Ant + CD + 0.05% CR and Ant + CD + 0.1% CR), two mortalities each were recorded on 2DPI. No diarrheal symptoms were observed in control groups (Negative control, CR control, Ant control and Ant+CR control).

3.2 Effect of CR supplementation on clinical score and body weight of *C. difficile* infected mice

Clinical scores of the individual animals in different groups were recorded using standard clinical score chart, from 1DPI to 7 DPI (Table. 2a and 2b) (Chen et al., 2008; Sun et al., 2011a). The *C. difficile* control group (Ant+CD group) exhibited significantly increased severity, as indicated by a higher average clinical score per group on 1, 2, 3 and 4 DPI ($p < 0.05$). The severity of CDAD in animals supplemented prophylactically with 0.05% and 0.1% CR (Ant + CD + 0.05% CR and Ant + CD + 0.1% CR groups) was lesser than that of the untreated group (Ant+CD) ($p < 0.05$). On 1DPI, there was a dose-dependent reduction in the severity of infection in CR supplemented groups. Irrespective of the treatments, all surviving morbid animals recovered by 6DPI, as indicated by a zero clinical score.

A similar trend was observed in average body weights of animals in different treatment groups. Body weights were recorded daily and the relative percentage weight with respect to the initial weight before *C. difficile* infection was calculated (Fig. 3). Carvacrol alone (CR control) and in combination with antibiotic (Ant+CR control) did not cause any significant weight loss compared to the negative control. All mice in the *C. difficile* control group (Ant+CD) showed significant and progressive weight loss from 1 DPI to 5DPI compared to the negative control ($p < 0.05$). However, CR-treated and *C. difficile* infected mice (Ant + CD + 0.05% CR and Ant + CD + 0.1% CR groups) showed a significantly lesser weight loss in comparison to untreated and

C. difficile infected group (Ant+CD) from 1 DPI and 2DPI, with regaining of the initial weight on 3DPI ($p<0.05$). No significant difference in recorded weight loss was observed between 0.05 and 0.1% CR-treated *C. difficile* infected mice except for a rapid and early increase in the body weight on 2DPI in the 0.05% CR group.

3.3 Effect of CR supplementation on the gut microbiome of *C. difficile* infected and non-infected mice.

Microbiome analysis results revealed specific patterns in the composition of different bacterial taxa in different treatment groups. In the phylum level, the gut microbiome of negative control mice was predominated by Bacterioidetes, followed by Firmicutes (Fig. 4) with a minimal proportion of other phyla, including Proteobacteria. A similar trend was observed in CR control group, with an abundance of Bacterioidetes followed by Firmicutes, although the proportion of Firmicutes was slightly higher than that of the negative control group. Antibiotic administration significantly increased the proportion of Proteobacteria in antibiotic-treated (Ant control) group compared to the negative control and CR control groups ($p<0.05$). Interestingly, supplementation of CR along with antibiotic (Ant + CR control) significantly reduced the abundance of Proteobacteria compared to the antibiotic only (Ant control) group. The *C. difficile* control group (Ant + CD), where *C. difficile* spores were orally gavaged after antibiotic treatment, exhibited a remarkably increased abundance of Proteobacteria along with a greater proportion of Verrucomicrobia compared to all other control groups (Negative control, CR control, Ant control, Ant+ CR control groups) ($p<0.05$). In addition, the abundance of Bacterioidetes and Firmicutes was significantly reduced in *C. difficile* control group (Ant+CD), compared to uninfected controls ($p<0.05$). Strikingly, this alteration in the abundance of Proteobacteria, Firmicutes, Bacterioidetes and Verrucobacteria due to *C. difficile* infection was reversed significantly by CR

supplementation, as observed in the CR-treated and *C. difficile* infected groups (Ant + CD + 0.05% CR and Ant + CD + 0.10% CR groups) ($p < 0.05$).

At the Order and Family level, an increased abundance of Enterobacteriaceae was observed in antibiotic alone (Ant control) and *C. difficile* (Ant+CD) groups compared to negative control and CR control groups (Fig. 5a). Moreover, CR supplementation significantly reduced the abundance of Enterobacteriaceae induced by the antibiotic administration and *C. difficile* infection, as indicated by a significant reduction in their abundance in Ant + CR control group, Ant + CD + 0.05% CR and Ant + CD + 0.10% CR groups ($p < 0.05$) (Fig. 5a). Carvacrol treatment significantly increased the abundance of Lactobacillaceae and Lachnospiraceae in the gut microbiome compared to that of negative control (Fig. 5b) ($p < 0.05$). The abundance of Lactobacillaceae and Lachnospiraceae was significantly reduced following antibiotic treatment (Ant control group) and *C. difficile* (Ant+CD control group) infection, compared to the Negative control and CR alone (CR control) groups ($p < 0.05$). This effect was significantly reversed by the supplementation of CR in Ant + CR control, Ant + CD + 0.5 CR and Ant + CD + 0.5 CR groups.

Inverse Simpson plot revealed a differential pattern of bacterial diversity in various treatment groups (Fig. 6). Strikingly, CR treatment did not alter the diversity of the gut bacterial community compared to the untreated control group (Negative control). As expected, antibiotic treatment significantly reduced the bacterial diversity compared to control and CR group. There was a marked reduction in the diversity of the bacterial community in *C. difficile* infected groups, irrespective of the CR treatment. Moreover, NMDS plot representing the relationships between samples in various treatment groups based on the abundance of species present in each sample revealed a close clustering of CR control samples and untreated control samples (Fig. 7). This

representation suggests that the species abundance in CR treatment groups is comparable to untreated mice indicating minimal effect of CR on gut microbial diversity.

4. Discussion

Prolonged antibiotic therapy and subsequent gut dysbiosis result in *C. difficile* spore germination, and colonization of the large intestine with vegetative cells of the bacterium, and subsequent production of toxins TcdA and TcdB, resulting in *C. difficile* associated diarrhea [1]. *C. difficile* toxins lead to intestinal inflammation, increased epithelial permeability (Castagliuolo et al., 1998; Feltis et al., 2000; He et al., 2002), enhanced cytokine and chemokine production (Castagliuolo et al., 1998; He et al., 2002), neutrophil infiltration (Kelly et al., 1994) and the release of reactive oxygen intermediates (He et al., 2002), thereby causing direct damage to the intestinal mucosa (Ng et al., 2010). Antibiotics are the primary line of treatment in *C. difficile* infection, although the use of antibiotics has been documented for inducing and aggravating gut dysbiosis and relapse of the infection post-therapy. In addition, increasing incidence of *C. difficile* acquiring antibiotic resistance is reported worldwide.

In the current study, we investigated the prophylactic efficacy of CR as an alternative antimicrobial agent that can ameliorate *C. difficile* associated diarrhea without inducing gut dysbiosis. Previous studies have reported that supplementation of low doses of CR exerted no detrimental effects on endogenous bacterial populations, including *Lactobacilli* and *Bifidobacteria* in pigs and poultry (Jamroz et al., 2005; Si et al., 2006).

Previous studies conducted in our laboratory revealed that SICs of CR reduced *C. difficile* toxin production and cytotoxicity in Vero cells *in vitro* (Mooyottu et al., 2014d). In addition, our previous experiments showed an inhibition of *C. difficile* spore outgrowth in the presence of CR (Mooyottu et al., 2014a). The results from the mice experiment indicated that our *in vitro* results

apparently well translated *in vivo* with regards to the clinical outcome and gut microbiome of the animals when prophylactically treated with CR prior to *C. difficile* infection. As expected, CR supplementation significantly reduced the incidence of diarrhea in *C. difficile* infected mice ($p < 0.05$). Moreover, CR supplementation significantly reduced the severity of clinical infection in *C. difficile* infected mice, as evident from a reduced average clinical score compared to the infected control group (Ant+CD) ($p < 0.05$). However, CR-treated and *C. difficile* infected mice exhibited significantly lesser weight loss compared to the untreated group (Ant+CD). The reduced severity of CDAD in CR-treated mice compared to infected control group (Ant+CD) could be attributed to an inhibitory effect of the phytochemical on *C. difficile* spore outgrowth and/or the toxin production, as observed in our *in vitro* studies. Moreover, CR has been reported to possess anti-inflammatory and anti-diarrheal properties (Baser, 2008), which could also have exerted a beneficial effect in *C. difficile* infected mice.

A healthy and normal gut microflora is crucial for preventing pathogen colonization and a variety of enteric bacterial infections, including *C. difficile* (Britton & Young, 2014). The most important predisposing factor for *C. difficile* infection is the disruption of normal gut microbiota (Hookman & Barkin, 2009). Antibiotic therapy significantly alters the microbial composition and diversity; and in many cases alterations in the microbial diversity persist to an extent, even after withdrawing antibiotic administration (Antonopoulos et al., 2009; Dethlefsen et al., 2008b). In human patients, as age advances, the protective bacterial population of Firmicutes considerably diminishes accompanied by an increase in Bacteroidetes and undesirable species of Proteobacteria in the gut (Biagi et al., 2010; Claesson et al., 2011; Hopkins et al., 2001b). Moreover, age-related senescence in the immune status of the elderly, along with frequent hospital visits during old age contribute to a detrimental alteration in the gut microbiome and subsequent colonization of *C.*

difficile (Seekatz & Young, 2014). Other important factors that detrimentally affect the gut microbiota and predispose *C. difficile* infection are the use of proton pump inhibitors and chronic gastrointestinal diseases (Berg et al., 2013; Dial et al., 2005; Vesper et al., 2009). Proton pump inhibitors alter the pH of the gut, thereby affecting the microbial population, especially beneficial bacteria such as *Lactobacillus* species (Altman et al., 2008; Vesper et al., 2009). In addition, disease conditions such as inflammatory bowel disease (IBD) induce significant gut dysbiosis, which reduces the diversity of the protective population of Firmicutes and Bacteroides population accompanied by an increased Proteobacteria in the gut of affected patients (Manichanh et al., 2006; Nagalingam & Lynch, 2012). Increased abundance of Verrucomicrobia has also been shown in patients with antibiotic-associated gut dysbiosis (Weingarden et al., 2014). Moreover, the paucity of Firmicutes, especially the depletion of Ruminococcaceae, Lachnospiraceae, and butyrogenic bacteria within this phylum observed in *C. difficile* infection and nosocomial diarrhea in humans (Antharam et al., 2013). Moreover, *C. difficile* infected patients have a higher count of Enterobacteriaceae (Proteobacteria) and decreased Enterococcaceae (Firmicutes) (Hopkins & Macfarlane, 2002; Rea et al., 2012; Schubert et al., 2014).

The changes in the gut microbiome diversity and alterations in the relative abundance of different bacterial communities in human patients are replicated in mice models of *C. difficile* infection (Semenyuk et al., 2015). Antibiotic treatment and subsequent *C. difficile* infection significantly reduced the abundance of Firmicutes and Bacteroides in phylum level. Similar trends are observed in all taxonomical level such as a reduction in protective Lactobacillaceae, Lachnospiraceae, and Bifidobacteriaceae. A dramatic increase in the abundance of Proteobacteria specifically Enterobacteriaceae has been found in the antibiotic treated and *C. difficile* infected

mice (Semenyuk et al., 2015). Similarly, antibiotic treatment and subsequent *C. difficile* infection significantly reduced microbiome diversity in mouse gut (Semenyuk et al., 2015).

In this study, CR treatment did not reduce the bacterial diversity in the mouse gut. To date, a majority of the antimicrobial compounds, especially antibiotics, have significantly altered the microbial diversity, and cause dysbiosis by changing the abundance of bacterial communities (Semenyuk et al., 2015). Moreover, CR treatment significantly increased the abundance of beneficial bacterial populations such as Firmicutes, specifically the members of Lactobacillaceae and Lachnospiraceae. In addition, CR treatment alone did not increase the abundance of detrimental bacterial populations compared to untreated control animals. Strikingly, CR reduced antibiotic-induced increases in the abundance of unfavorable bacterial populations such as Proteobacteria, specifically pathogenic gamma proteobacteria, including Enterobacteriaceae and other bacterial populations such as Verrucobacteria (Fig. 5a). Surprisingly, this beneficial shift brought about by CR treatment in the gut microbiome of antibiotic-treated and *C. difficile* infected animals is very much similar to that of human patients who have undergone fecal microbiome transplantation (Weingarden et al., 2014), which is documented as one of the most effective strategies against severe *C. difficile* infection (Ofosu, 2016b; Schenck et al., 2015). These results suggest that reduced or delayed clinical infection rate and less severe clinical presentation of CR-treated animals could be attributed in part to the beneficial shift in the gut microbiome.

To conclude, our results suggest CR supplementation to be protective against *C. difficile* infection in mice. Carvacrol supplementation significantly reduced the incidence of diarrhea and mitigated the severity of *C. difficile* induced clinical symptoms, inducing a favorable shift in the composition of the gut microbiota without detrimentally affecting the gut microbiome diversity in

mice. These findings suggest the potential of CR as an anti- *C. difficile* agent, however, further clinical studies are warranted to confirm this.

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Table 1. Different treatment groups used in the experiment:

Group	Antibiotic	CR	CD
Negative control	-	-	-
Ant + CD	+	-	+
Ant + CR	+	0.1%	-
CR control	-	0.1%	-
Ant + CD + CR 0.05%	+	0.05%	+
Ant + CD + CR 0.1%	+	0.1%	+
Ant control	+	-	-

Abbreviations: Ant (Antibiotic), CD (*C. difficile*), CR (Carvacrol)

Table 2a. Mouse clinical score sheet:

MOUSE CLINICAL SCORE SHEET				
	0	1	3	5
Coat	Normal	Lack of grooming	Rough/Ruffled fur	
Activity	Normal	Isolated, abnormal posture	Huddled/inactive	Moribund, unresponsive or seizing
Breathing	Normal	Rapid, shallow	Rapid, abdominal	Laboured, blue
Movement	Normal	Slight incoordination/ abnormality	Incoordinated, walking of toes, reluctant to move	Staggering, paralysis, lack of mobility
Condition	BC 3	BC 2+	BC 2	BC 1
Dehydration	Nil	Skin less elastic	Skin tents	Skin tents, eyes sunken
Feces	Normal	Moist faeces	Diarrhoea or dry faeces	Uncontrolled diarrhoea, Wet tail
Body Weight	Normal	Markedly reduced growth (e.g. severe runting) 5% over 24 hours	Weight loss up to 10% over 24 hours	Weight loss >10% over 24 hours or >20% over time
<ul style="list-style-type: none"> • Moribund animals (A lack of responsiveness to manual stimulation, lack of mobility or inability or failure to eat or drink or a clinical score > 25) will be euthanized. 				

Table 2b. Mouse body condition chart

	<p>BC 1</p> <p>Mouse is emaciated.</p> <ul style="list-style-type: none"> ◦ <i>Skeletal structure extremely prominent; little or no flesh cover.</i> ◦ <i>Vertebrae distinctly segmented.</i>
	<p>BC 2</p> <p>Mouse is underconditioned.</p> <ul style="list-style-type: none"> ◦ <i>Segmentation of vertebral column evident.</i> ◦ <i>Dorsal pelvic bones are readily palpable.</i>
	<p>BC 3</p> <p>Mouse is well-conditioned.</p> <ul style="list-style-type: none"> ◦ <i>Vertebrae and dorsal pelvis not prominent; palpable with slight pressure.</i>
	<p>BC 4</p> <p>Mouse is overconditioned.</p> <ul style="list-style-type: none"> ◦ <i>Spine is a continuous column.</i> ◦ <i>Vertebrae palpable only with firm pressure.</i>
	<p>BC 5</p> <p>Mouse is obese.</p> <ul style="list-style-type: none"> ◦ <i>Mouse is smooth and bulky.</i> ◦ <i>Bone structure disappears under flesh and subcutaneous fat.</i>
<p>A "+" or a "-" can be added to the body condition score if additional increments are necessary (i.e. ...2+, 2, 2-...)</p>	

Figures

Fig. 1. Effect of CR supplementation on the incidence of *C. difficile* associated diarrhea in mice

Five to six-week-old C57BL/6 mice were randomly divided into seven treatment groups of 8 mice each. Mice were fed with irradiated feed supplemented with CR (0%, 0.05%, and 0.1%); the challenge groups were made susceptible to *C. difficile* by administering an antibiotic cocktail in water and an intra-peritoneal injection of clindamycin. Further, challenge and control groups were infected with 10^5 CFU/ml of a hypervirulent *C. difficile* (ATCC 1870) spores or PBS and observed for clinical signs for ten days. Respective control groups for CR, antibiotics, and their combination were included for investigating their effect on mouse enteric microflora. The incidence of clinical signs including diarrhea was recorded from 1DPI to 7DPI. Groups: 1) Negative Control: Mice treated with no CR, no antibiotics and no *C. difficile* 2) CR control: Mice fed with 0.1% CR in feed, 3) Ant Control: Mice administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 4) Ant + CR Control: Mice fed with CR (0.1%) supplemented feed and administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 5) Ant+CD: administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected by *C. difficile* 6) (Ant + CD + 0.05% CR): Mice fed with CR (0.05%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*) (Ant + CD + 0.1% CR): Mice fed with CR (0.1%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*.

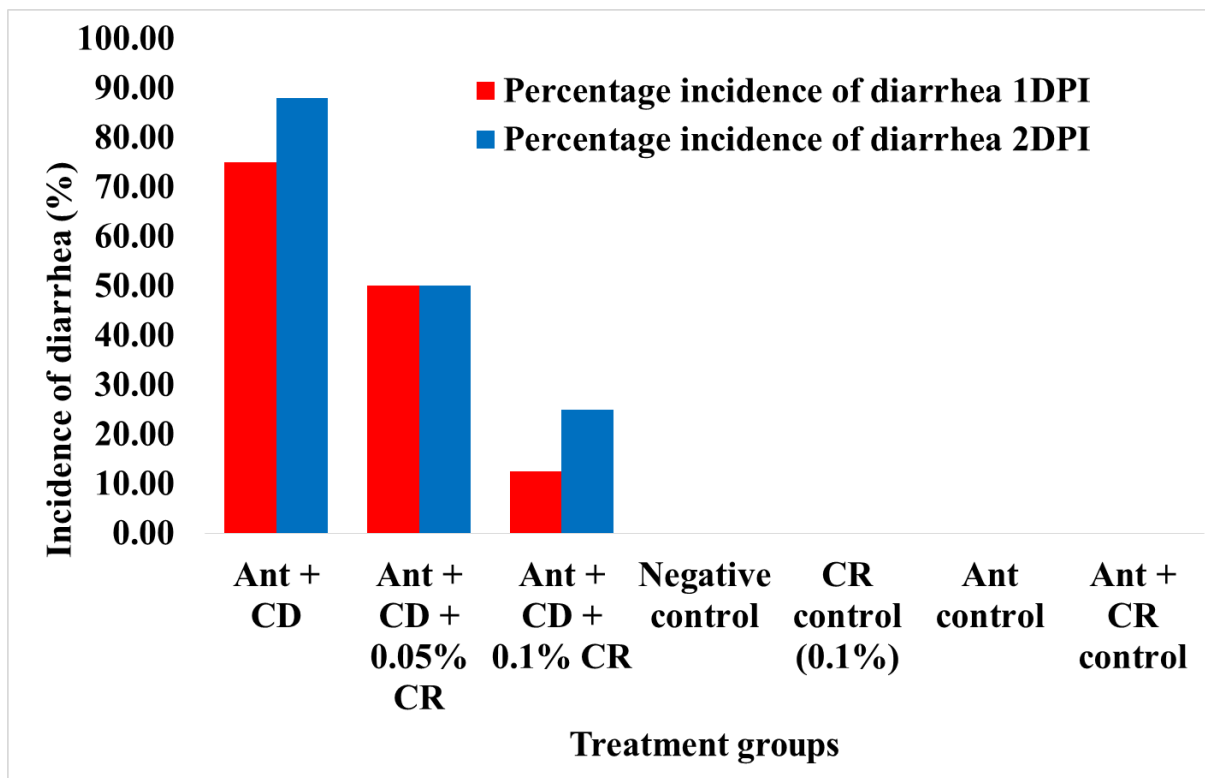


Fig. 2. Effect of CR supplementation on the severity of CD associated disease in mice

Five to six-week-old C57BL/6 mice were randomly divided into seven treatment groups of 8 mice each. Mice were fed with irradiated feed supplemented with CR (0%, 0.05%, and 0.1%); the challenge groups were made susceptible to *C. difficile* by administering an antibiotic cocktail in water and an intra-peritoneal injection of clindamycin. Further, challenge and control groups were infected with 10^5 CFU/ml of a hypervirulent *C. difficile* (ATCC 1870) spores or PBS and observed for clinical signs for ten days. Respective control groups for CR, antibiotics, and their combination were included for investigating their effect on mouse enteric microflora. The mice were monitored for signs of *C. difficile* infection such as diarrhea, hunched posture and wet tail using a mouse clinical score sheet. Groups: 1) Negative Control: Mice treated with no CR, no antibiotics and no *C. difficile* 2) CR control: Mice fed with 0.1% CR in feed, 3) Ant Control: Mice administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 4) Ant + CR Control: Mice fed with CR (0.1%) supplemented feed and administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 5) Ant+CD: administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected by *C. difficile* 6) (Ant + CD + 0.05% CR): Mice fed with CR (0.05%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*) (Ant + CD + 0.1% CR): Mice fed with CR (0.1%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*.

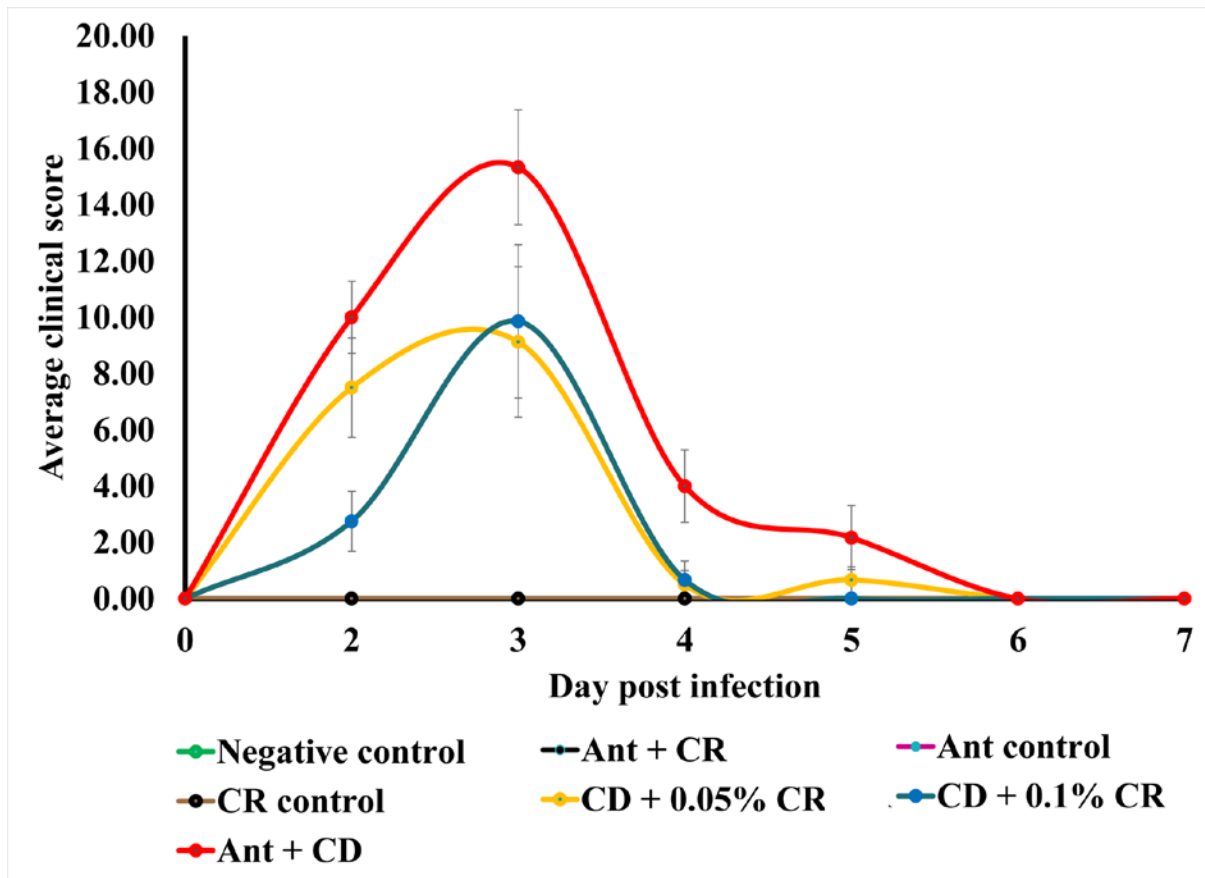


Fig. 3. Effect of CR supplementation on relative weight loss in *C. difficile* infected and non-infected mice

Five to six-week-old C57BL/6 mice were randomly divided into seven treatment groups of 8 mice each. Mice were fed with irradiated feed supplemented with CR (0%, 0.05%, and 0.1%); the challenge groups were made susceptible to *C. difficile* by administering an antibiotic cocktail in water and an intra-peritoneal injection of clindamycin. Further, challenge and control groups were infected with 10^5 CFU/ml of a hypervirulent *C. difficile* (ATCC 1870) spores or PBS and observed for clinical signs for ten days. Respective control groups for CR, antibiotics, and their combination were included for investigating their effect on mouse enteric microflora. The body weights of the animals were recorded daily and the relative percentage weight with respect to the initial weight prior to the infection was calculated. Groups: 1) Negative Control: Mice treated with no CR, no antibiotics and no *C. difficile* 2) CR control: Mice fed with 0.1% CR in feed, 3) Ant Control: Mice administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 4) Ant + CR Control: Mice fed with CR (0.1%) supplemented feed and administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 5) Ant+CD: administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected by *C. difficile* 6) (Ant + CD + 0.05% CR): Mice fed with CR (0.05%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*) (Ant + CD + 0.1% CR): Mice fed with CR (0.1%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*.

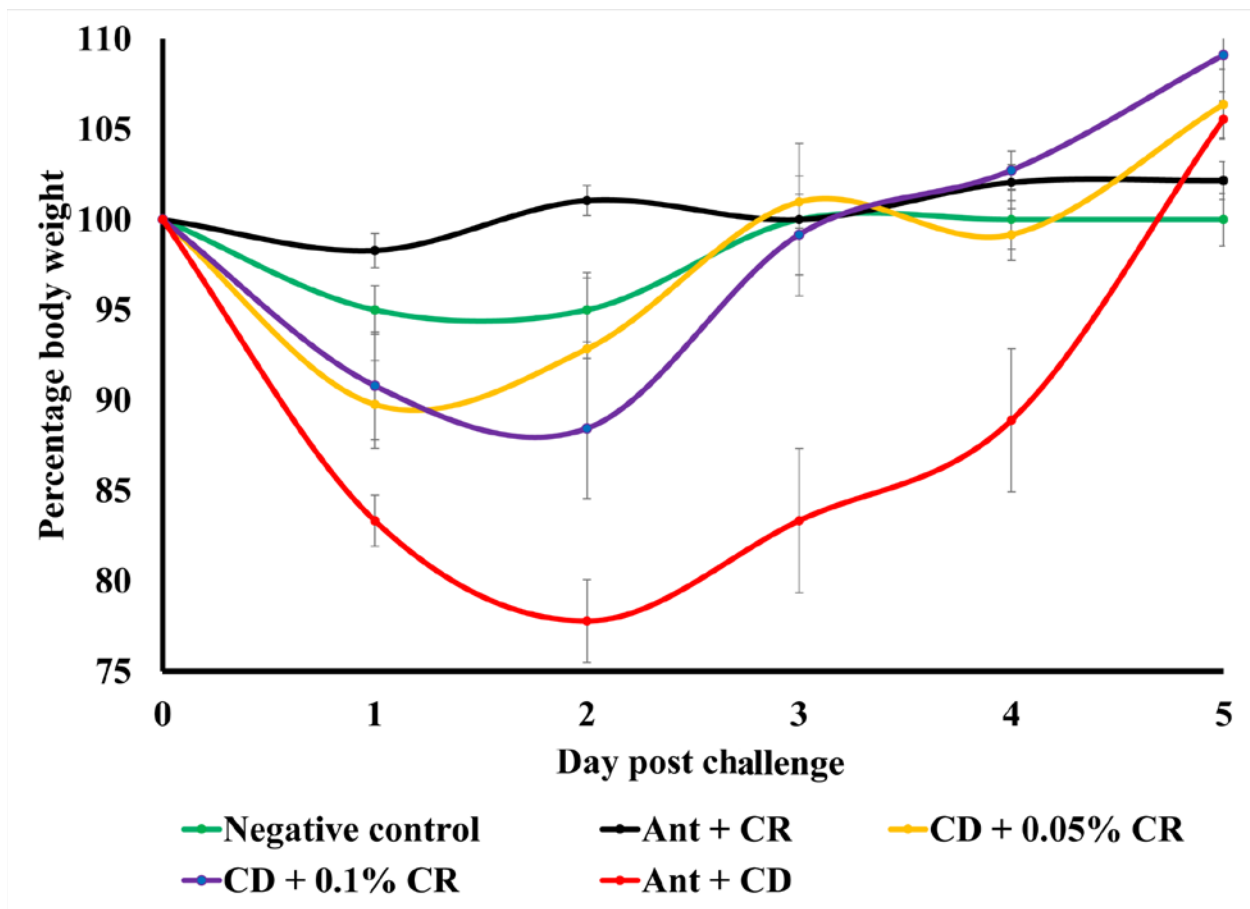


Fig. 4. Effect of CR supplementation on the abundance of major gut microbiota (phyla level) in the antibiotic treated and *C. difficile* challenged mice

Five to six-week-old C57BL/6 mice were randomly divided into seven treatment groups of 8 mice each. Mice were fed with irradiated feed supplemented with CR (0%, 0.05%, and 0.1%); the challenge groups were made susceptible to *C. difficile* by administering an antibiotic cocktail in water and an intra-peritoneal injection of clindamycin. Further, challenge and control groups were infected with 10^5 CFU/ml of a hypervirulent *C. difficile* (ATCC 1870) spores or PBS and observed for clinical signs for ten days. Respective control groups for CR, antibiotics, and their combination were included for investigating their effect on mouse enteric microflora. The fecal samples were collected 2 DPI from which DNA was extracted for microbiome analysis using Illumina MiSeq platform, and the relative abundance of OTUs of major phyla, order, family, and genera was determined using microbiome analysis. Groups: 1) Negative Control: Mice treated with no CR, no antibiotics and no *C. difficile* 2) CR control: Mice fed with 0.1% CR in feed, 3) Ant Control: Mice administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 4) Ant + CR Control: Mice fed with CR (0.1%) supplemented feed and administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 5) Ant+CD: administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected by *C. difficile* 6) (Ant + CD + 0.05% CR): Mice fed with CR (0.05%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*) (Ant + CD + 0.1% CR): Mice fed with CR (0.1%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*.

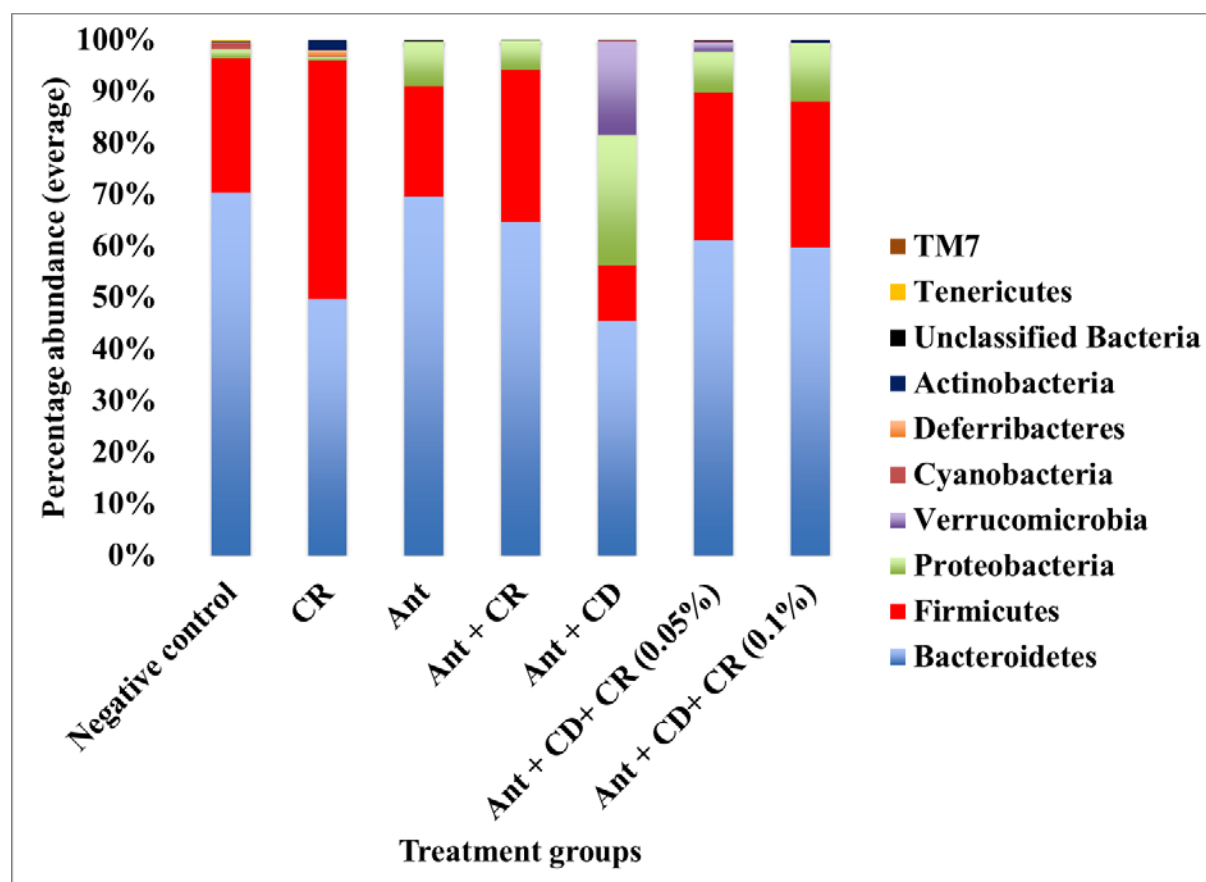


Fig. 5a & 5b. Effect of CR supplementation on the abundance of Enterobacteriaceae (5a), Lactobacillaceae (5b) and Lachnospiraceae (5b) in the antibiotic treated and *C. difficile* challenged mice

Five to six week-old C57BL/6 mice were randomly divided into 7 treatment groups of 8 mice each. Mice were fed with irradiated feed supplemented with CR (0%, 0.05%, and 0.1%); the challenge groups were made susceptible to *C. difficile* by administering an antibiotic cocktail in water and an intra-peritoneal injection of clindamycin. Further, challenge and control groups were infected with 10⁵ CFU/ml of a hypervirulent *C. difficile* (ATCC 1870) spores or PBS, and observed for clinical signs for 10 days. Respective

control groups for CR, antibiotics and their combination were included for investigating their effect on mouse enteric microflora. The fecal samples were collected 2 DPI from which DNA was extracted for microbiome analysis using Illumina MiSeq platform and the relative abundance of OTUs at different taxonomic level (Family. Enterobacteriaceae (Fig.5a), Lactobacillaceae and Lachnospiraceae (Fig. 5b) was determined using microbiome analysis. Groups: 1) Negative Control: Mice treated with no CR, no antibiotics and no *C. difficile* 2) CR control: Mice fed with 0.1% CR in feed, 3) Ant Control: Mice administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 4) Ant + CR Control: Mice fed with CR (0.1%) supplemented feed and administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 5) Ant+CD: administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected by *C. difficile* 6) (Ant + CD + 0.05% CR): Mice fed with CR (0.05%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile* 7) (Ant + CD + 0.1% CR): Mice fed with CR (0.1%),

administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*. (Treatments significantly differed from the negative control ($p < 0.05$))

Fig. 5a

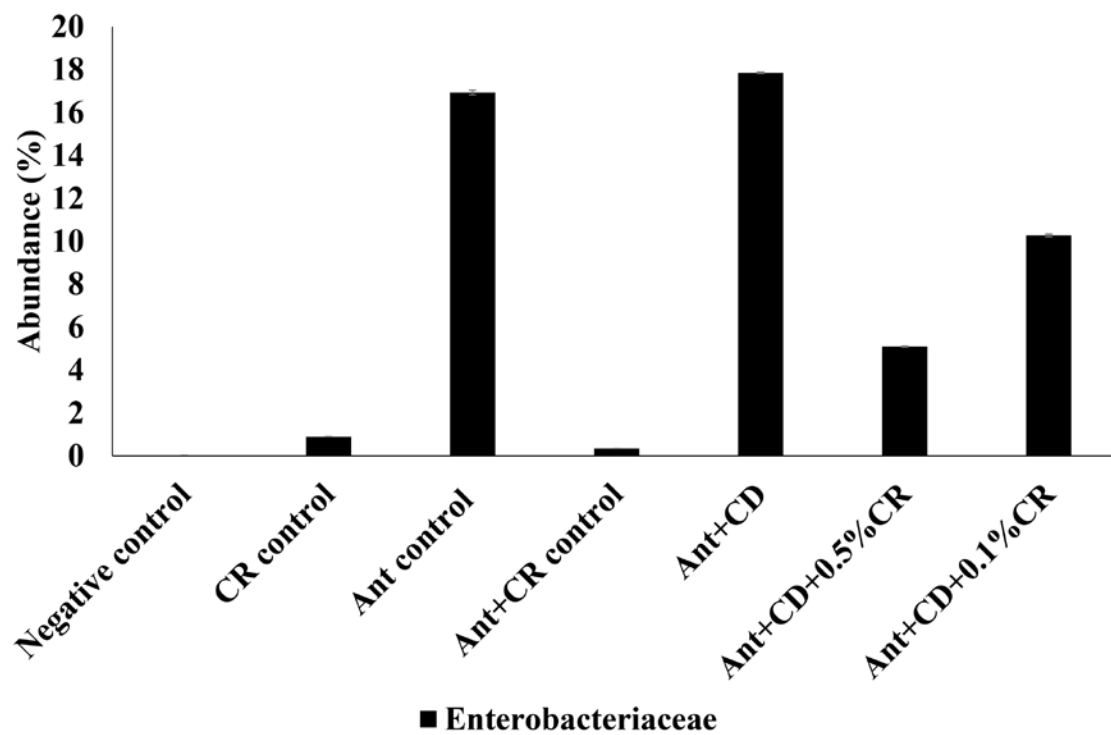


Fig. 5b

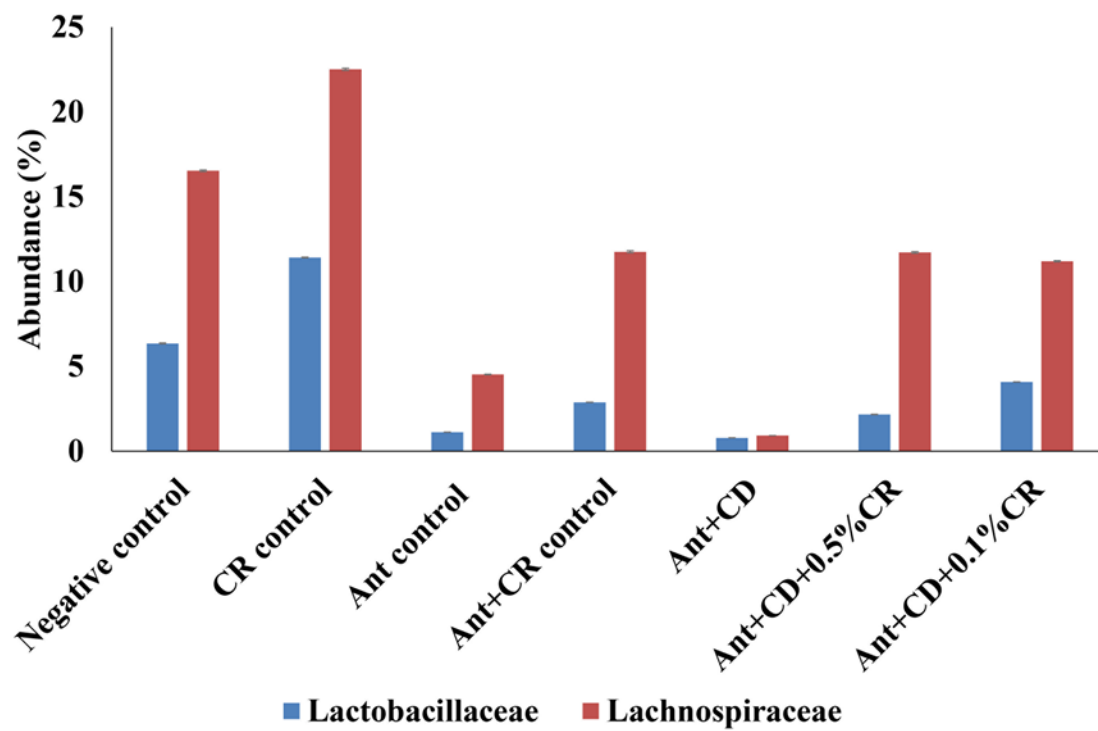


Fig. 6. Effect of CR supplementation on the diversity of gut microbiota of antibiotic treated and *C. difficile* challenged mice

Five to six week-old C57BL/6 mice were randomly divided into 7 treatment groups of 8 mice each. Mice were fed with irradiated feed supplemented with CR (0%, 0.05%, and 0.1%); the challenge groups were made susceptible to *C. difficile* by administering an antibiotic cocktail in water and an intra-peritoneal injection of clindamycin. Further, challenge and control groups were infected with 10^5 CFU/ml of a hypervirulent *C. difficile* (ATCC 1870) spores or PBS, and observed for clinical signs for 10 days. Respective control groups for CR, antibiotics and their combination were included for investigating their effect on mouse enteric microflora. The fecal samples were collected 2 DPI from which DNA was extracted for microbiome analysis using Illumina MiSeq platform, and Alpha diversity was calculated by using inverse Simpson to measure the richness and evenness of the OTUs. Groups: 1) Negative Control: Mice treated with no CR, no antibiotics and no *C. difficile* 2) CR control: Mice fed with 0.1% CR in feed, 3) Ant Control: Mice administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 4) Ant + CR Control: Mice fed with CR (0.1%) supplemented feed and administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 5) Ant+CD: administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected by *C. difficile* 6) (Ant + CD + 0.05% CR): Mice fed with CR (0.05%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile* 7) (Ant + CD + 0.1% CR): Mice fed with CR (0.1%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*.

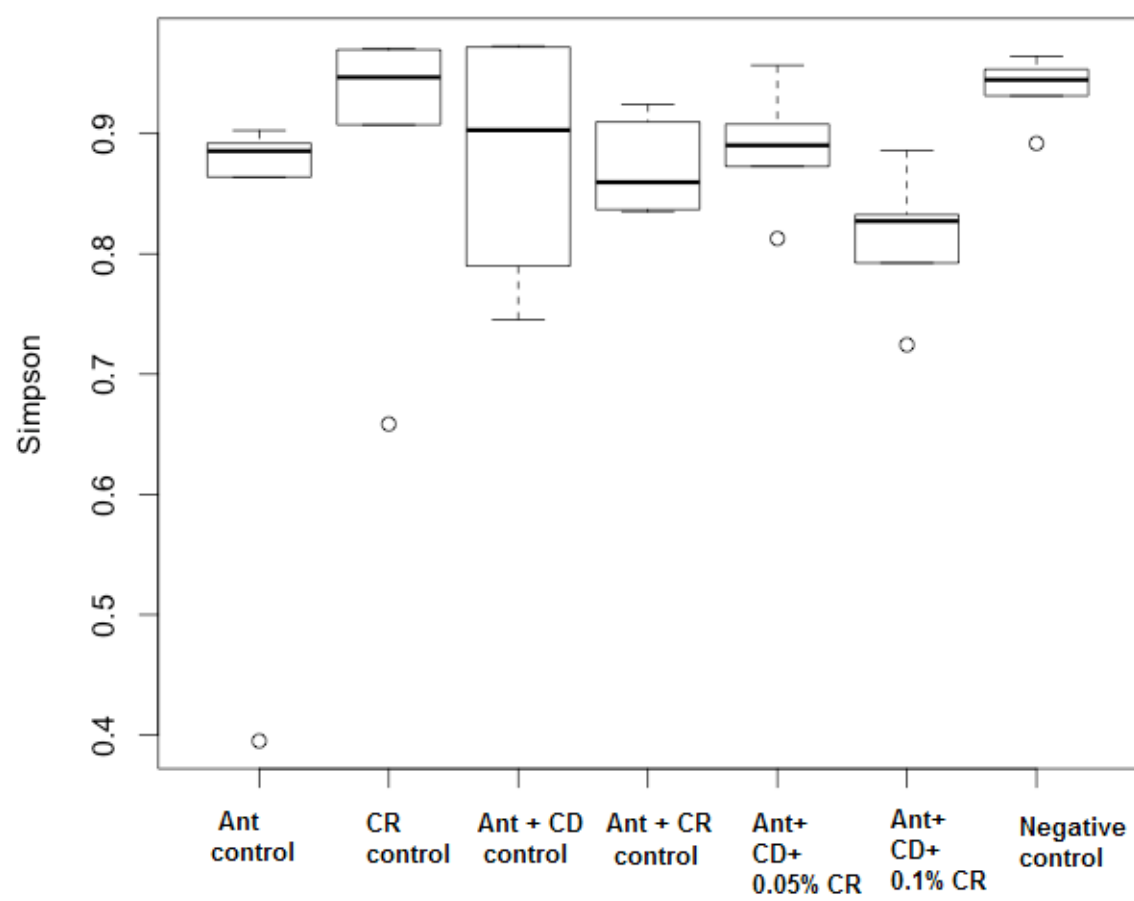
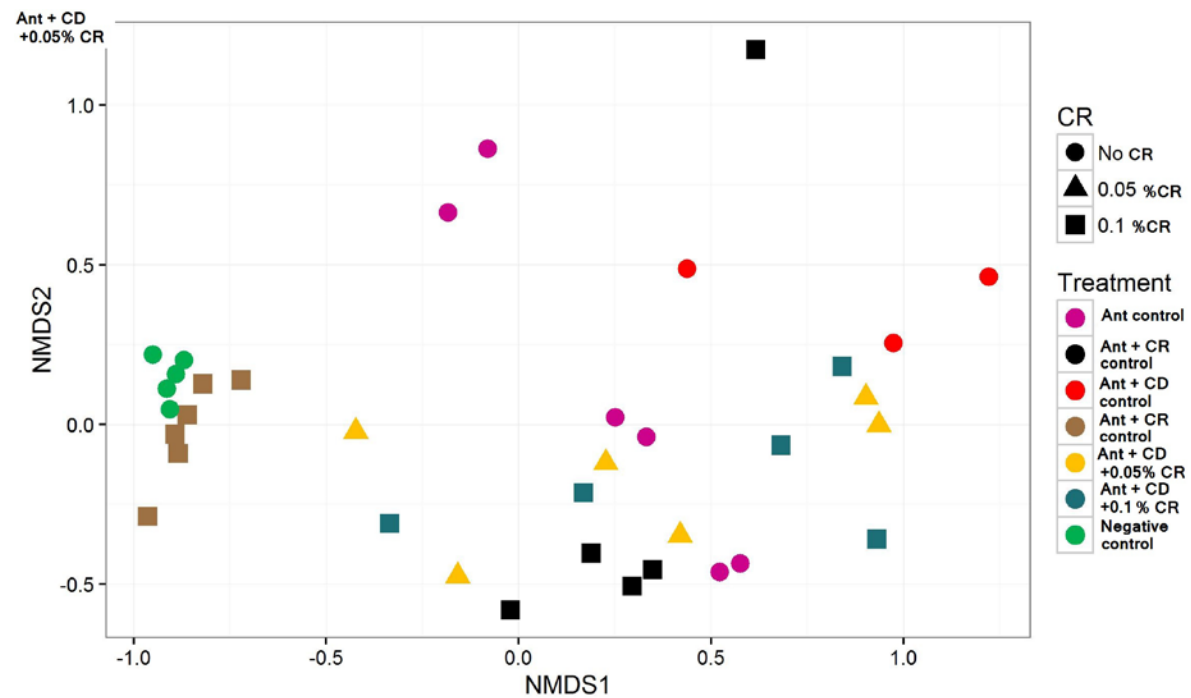


Fig. 7. Effect of CR supplementation on the diversity of gut microbiota of antibiotic treated and *C. difficile* challenged mice

Five to six week-old C57BL/6 mice were randomly divided into 7 treatment groups of 8 mice each. Mice were fed with irradiated feed supplemented with CR (0%, 0.05%, and 0.1%); the challenge groups were made susceptible to *C. difficile* by administering an antibiotic cocktail in water and an intra-peritoneal injection of clindamycin. Further, challenge and control groups were infected with 10^5 CFU/ml of a hypervirulent *C. difficile* (ATCC 1870) spores or PBS, and observed for clinical signs for 10 days. Respective control groups for CR, antibiotics and their combination were included for investigating their effect on mouse enteric microflora. The fecal samples were collected 2 DPI from which DNA was extracted for microbiome analysis using Illumina MiSeq platform. Relationships between treatment groups based on the abundance of species present in each sample were plotted. NMS ordinations were run in R (v 3.3.0) using metaMDS in the vegan (v 2.3-5) package after calculating the stress scree plots to determine the number of axes required to achieve stress below 0.2, plotted using ggplot2 (v 2.1.0). Groups: 1) Negative Control: Mice treated with no CR, no antibiotics and no *C. difficile* 2) CR control: Mice fed with 0.1% CR in feed, 3) Ant Control: Mice administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 4) Ant + CR Control: Mice fed with CR (0.1%) supplemented feed and administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, 5) Ant+CD: administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected by *C. difficile* 6) (Ant + CD + 0.05% CR): Mice fed with CR (0.05%), administered with antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile* 7) (Ant + CD + 0.1% CR): Mice fed with CR (0.1%), administered with

antibiotic cocktail in water and an intra-peritoneal injection of clindamycin, and infected with *C. difficile*.



Chapter VII

Summary

Clostridium difficile is a nosocomial pathogen that causes a toxin-mediated enteric disease in human. More than 300,000 cases of *C. difficile*-associated disease (CDAD) are reported annually in the United States, resulting in more than US\$3 billion as healthcare costs. According to US Department of Health and Human Services, the New England region, including Connecticut reported the highest rate of *C. difficile* associated hospitalizations in recent years. *C. difficile* mostly affects long-term hospital inpatients and the elderly undergoing prolonged antibiotic therapy. Prolonged antibiotic therapy results in gut dysbiosis, leading to the germination of *C. difficile* spores and pathogen colonization in the intestine with subsequent production of toxins. *C. difficile*, TcdA, and TcdB, cause severe inflammatory response with the release of cytokines and leukotrienes, leading to pseudomembrane formation in the intestine and watery diarrhea.

The incidence and severity of the disease associated with *C. difficile* have increased in the US with the emergence of hypervirulent strains and community associated outbreaks. The detection of genotypically similar and identical *C. difficile* strains implicated in human infections from foods and food animals indicate the potential role of food as a source of community associated *C. difficile* disease. In this study, we investigated the prevalence of *C. difficile* spores in retail meat sold in Connecticut. One hundred samples each of ground beef, pork and chicken obtained from geographically distant grocery stores in Connecticut were tested for *C. difficile*. Positive isolates were characterized by ribotyping, antibiotic susceptibility, toxin production and whole genome sequencing. Of the 300 meat samples, only two pork samples tested positive for *C. difficile* indicating a very low prevalence of *C. difficile* in meat. The isolates were non-toxigenic, but found to have an array of antibiotic resistance genes and mobile elements, which can potentially contribute to the generation of multi-drug resistant toxigenic strains.

Although exposure to broad-spectrum antibiotics predisposes patients to *C. difficile* associated disease (CDAD) by disrupting the normal gut flora, antibiotics are still the primary line of treatment against the disease. In addition, the emergence of antibiotic resistance in hypervirulent strains of *C. difficile* is increasingly reported worldwide, which raises concerns on the success of antibiotic treatment. Since *C. difficile* toxins are the major virulence factors responsible for the pathogenesis of CDAD, reducing *C. difficile* toxin production could significantly minimize its pathogenicity and improve disease outcome in humans. Therefore, the second objective of this research work investigated the efficacy of two, food-grade, plant-derived compounds, namely *trans*-cinnamaldehyde (TC) and carvacrol (CR) in reducing *C. difficile* toxin production and cytotoxicity *in vitro*. The effect of CR and TC on toxin production genes in a *codY* mutant and wild type *C. difficile* was also investigated. Carvacrol and TC substantially reduced *C. difficile* toxin production and cytotoxicity on Vero cells. The plant compounds also significantly down-regulated toxin production genes. Carvacrol and TC did not inhibit toxin production in the *codY* mutant of *C. difficile*, suggesting a potential *codY*-mediated anti-toxigenic mechanism of the plant compounds. In addition, this study revealed that the antitoxigenic concentrations of CR and TC did not inhibit the growth of beneficial gut bacteria. The results from this study suggest the potential use of TC and CR to attenuate *C. difficile* virulence

Although toxins are the major virulence factors responsible for the pathogenesis of *C. difficile* infection, spore formation, germination and outgrowth in *C. difficile* are critical for transmission and relapse of the disease. Spore production is a complex process in *C. difficile*, regulated by a set of genes, including *spo0A*, which is the master regulator of sporulation initiation along with associated kinases and different sigma factors such as *sigH*. Spores are shed in the feces, which contaminate hospitals and healthcare facilities causing infection through faeco-oral

route or recurrence of *C. difficile* associated disease in patients following germination of spores. Therapeutic agents that are capable of reducing *C. difficile* spore production could significantly minimize the transmission and relapse of *C. difficile* infections. Therefore, our third objective investigated the ability of carvacrol to reduce *C. difficile* spore production, germination and spore outgrowth. Moreover, the effect of CR on *C. difficile* sporulation genes was investigated using real-time qPCR. Carvacrol significantly reduced sporulation in *C. difficile* and down-regulated critical genes involved in spore production. Carvacrol did not inhibit *C. difficile* spore germination, however, it completely inhibited spore outgrowth. The results from this study suggest that CR could potentially be used to control spread and relapse of *difficile* by reducing spore production and outgrowth.

To validate the results from the second and third objectives of this study, a mouse experiment was conducted to investigate the effect of CR supplementation on *C. difficile* infection *in vivo*. Carvacrol supplementation significantly reduced the incidence of diarrhea and improved the clinical and diarrhea scores in mice ($p < 0.05$). Microbiome analysis revealed a significant increase in Proteobacteria and reduction in protective bacterial flora in antibiotic-treated and *C. difficile* -infected mice compared to control ($p < 0.05$). However, CR supplementation positively altered the microbiome composition, as revealed by an increased abundance of beneficial bacteria, including Firmicutes, and significantly reduced the proportion of detrimental flora such as Proteobacteria, without significantly affecting the gut microbiome diversity. Our results suggest that CR could potentially be used to control gut dysbiosis and reduce *C. difficile* infection.

To conclude, the results of this Ph.D. research work indicate the therapeutic potential of CR and TC against *C. difficile* infection in humans. In addition, this study confirmed that *C. difficile* occurs at very low levels in retail meat sold in Connecticut.